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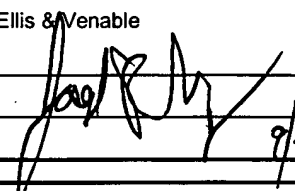
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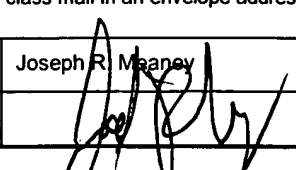
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	Filing Date	6/30/2000
	First Named Inventor	Michael F. Murray
	Art Unit	1617
	Examiner Name	Travers, Russel S.
Total Number of Pages in This Submission	Attorney Docket Number	PHJM0609-003

ENCLOSURES (check all that apply)		
<input type="checkbox"/> Fee Transmittal Form <input type="checkbox"/> Fee Attached <input type="checkbox"/> Amendment / Reply <input type="checkbox"/> After Final <input type="checkbox"/> Affidavits/declaration(s) <input type="checkbox"/> Extension of Time Request <input type="checkbox"/> Express Abandonment Request <input type="checkbox"/> Information Disclosure Statement <input type="checkbox"/> Certified Copy of Priority Document(s) <input type="checkbox"/> Response to Missing Parts/ Incomplete Application <input type="checkbox"/> Response to Missing Parts under 37 CFR 1.52 or 1.53	<input type="checkbox"/> Drawing(s) <input type="checkbox"/> Licensing-related Papers <input type="checkbox"/> Petition <input type="checkbox"/> Petition to Convert to a Provisional Application <input type="checkbox"/> Power of Attorney, Revocation Change of Correspondence Address <input type="checkbox"/> Terminal Disclaimer <input type="checkbox"/> Request for Refund <input type="checkbox"/> CD, Number of CD(s) _____	<input type="checkbox"/> After Allowance communication to Group <input type="checkbox"/> Appeal Communication to Board of Appeals and Interferences <input checked="" type="checkbox"/> Appeal Communication to Group (Appeal Notice, Brief, Reply Brief) <input type="checkbox"/> Proprietary Information <input type="checkbox"/> Status Letter <input type="checkbox"/> Other Enclosure(s) (please identify below):
Remarks Attached is Applicant's revised Appeal Brief for Application Number 09/809,552 that complies with 37 CFR 1.192(c) as required by the office communication of 8/11/2004.		

SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT	
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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Murray, Michael F.

Art Group: 1617

Serial No.: 09/609,552

Examiner: Russell Travers, J.D. Ph.D

Filed: 6/30/2000

Atty. Docket No.: PHJM0609-001

For: Treatment of Retrovirus Induced Derangements with Niacin Compounds

Mail Stop Appeal Brief - Patents
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Response to Office Communication Dated 8/11/2004

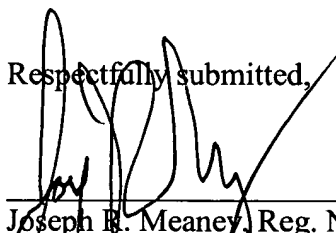
Dear Examiner:

This paper is responsive the Notification of Non-Compliance with 37 CFR 1.192(c) mailed 8/11/2004. As requested, Applicant has enclosed, in triplicate, a complete new brief that Applicant believes complies with 37 CFR 1.192(c), including a concise statement of the issues presented for review and identification of the claims that stand and fall together.

Applicant believes it has responded to every issue or point raised in the Examiner's office action.

Respectfully submitted,

Date: September 3, 2004



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JFW AF 1617

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Michael F. Murray, M.D.	Art Group: 1617
Serial No.: 09/609,552	Examiner: R.S. Travers, JD, Ph. D
Filed: June 30, 2000	Atty. Docket No.: PHJM0609-003
For: Treatment of Retrovirus Induced Derangements	

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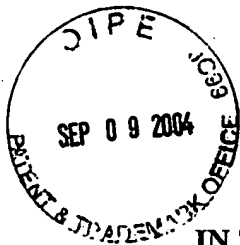
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- Appeal Brief (44 pages)(triplicate)
- Declaration of Michael F, Murray, M.D. (triplicate)
- Declaration of David G. Sidebottom, M.D. (triplicate)

Date: September 3, 2004



Joseph R. Meaney



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**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES**

In re Application of: Murray, Michael F.

Serial No.: 09/609,552

Filed: 6/30/2000

For: Treatment of Retrovirus Induced
Derangements with Niacin Compounds

Atty. Docket No.: PHJM0609-001

Technology Center: 3600

Group Art Unit: 1617

Examiner: Russell Travers, J.D. PhD

Appeal No.

APPELLANT'S BRIEF

This is an appeal from the final rejection of the Examiner dated October 30, 2003 rejecting Claims 1, 14-23, and 25-33. The requisite fee set forth in 37 CFR §1.17 is filed herewith.

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REAL PARTY IN INTEREST (37 C.F.R. 1.192(C)(1))

Foundation For Innovative Therapies, Inc., a Massachusetts non-profit corporation, is the real party in interest.

RELATED APPEALS AND INTERFERENCES (37 C.F.R. 1.192(C)(2))

There are no related appeals or interferences.

STATUS OF CLAIMS (37 C.F.R. 1.192(C)(3))

Applicant filed a non-provisional application with twenty-four claims (24) claims: nine independent claims and fifteen multiple dependent claims.

Examiner rejected all claims in the first Office Action. *See* Office Action dated July 27, 2001.

Applicant responded to the first Office Action through argument and amended the nine independent claims. *See* Applicant's response dated November 26, 2001,

Examiner rejected all of Applicant's argument in a second (and final) Office Action. *See* Office Action dated April 22, 2002.

Applicant responded to the final rejection on September 23, 2002 by filing a Request for Continued Examination.

Examiner replied on December 18, 2002 rejecting all claims.

Applicant responded on June 18, 2003 through argument and claim amendment.

Examiner replied on September 23, 2003 rejecting all claims.

Applicant filed a notice of appeal on March 18, 2004.

The status of the claims is as follows:

Allowed claims - none

Objected claims - none

Rejected claims - 1, 14-23, and 25-33

STATUS OF AMENDMENTS (37 C.F.R. 1.192(C)(4))

Claims 1, 14-23, and 25-33 are pending in the application. Examiner has rejected claims 1, 14-23, and 25-33.

SUMMARY OF THE INVENTION (37 C.F.R. 1.192(C)(5))

Applicant discovered that administering large daily doses of niacin to a person who is already receiving adequate daily intake of both niacin and tryptophan can raise a patient's level of systemic tryptophan. Prior to Applicant's discovery no one had known niacin could have this effect even though (1) the harmful effects of low levels of tryptophan have been documented for decades and (2) niacin has been available since 1937. Broadly, as set forth in claim 1, the present invention resides in a method of increasing systemic tryptophan comprising the administration of an effective amount of niacin for increasing systemic tryptophan to a patient in need of an increase in systemic tryptophan wherein the patient is infected with a retrovirus and wherein the patient has a diet that includes at least the RDA [recommended daily allowance] of niacin and tryptophan. (Specification, p. 6-9). More narrowly, the invention resides in a method of increasing systemic tryptophan of humans comprising the step of administering 3 grams of niacin daily to a human patient to increase systemic tryptophan wherein the human patient has a plasma tryptophan level of 91 micromol per liter or less. (Specification, p. 9-10).

ISSUES (37 C.F.R. 1.192(C)(6))

Examiner rejected claims 1, 14-23 and 25-33 under 35 U.S.C. § 112, first paragraph as failing to adequately teach how to make and/or use the invention, and thereby failing to provide an enabling disclosure. Examiner rejected claims 1, 14-23 and 25-33 under 35 U.S.C. § 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which the applicant regards as the invention. Examiner rejected claims 1, 14-21, 23, 24 and 26-33 under 35 U.S.C. § 103 as being obvious over Tang et al, Brown, et al, in view of Murray et al, all of record. Examiner rejected claims 22 and 25 under 35 U.S.C. § 103 obvious over Tang et al, Brown, et al, in view of Murray et al, all of record, in further view of Rideout et al. As such the following issues are presented on appeal.

Claim Rejections - 35 U.S.C. § 112 – First and Second Paragraphs

1. Do the working examples provided in the specification preclude a finding of non-enablement --at least for the examples shown?
2. Can Examiner's requirement of "exhaustive examples" that "define a class of compounds" be proper after the Federal Circuit expressly rejected the need for Applicants to provide exhaustive examples of controlled human testing?
3. By failing to provide support for why one would not expect to be able to extrapolate the four examples over the entire scope of the claims, can Examiner's enablement rejection be proper?
4. Can Examiner maintain an unsupported enablement rejection in light of the following evidence:
 - (a) No experimentation is needed to make or use the invention based on the content of the disclosure;
 - (b) The specification contains within it a connotation of how to use the invention;
 - (c) The direction provided by the inventor enables one skilled in the art to practice

the invention;

- (d) The state of the prior art favors enablement;
 - (e) The relative skill of those in the art favors enablement -- the art recognizes that standard modes of administration are known and contemplated; and,
 - (f) The breadth of the claims favor enablement?
5. By disclosing at least one method for making and using the claimed invention that bears a reasonable correlation to the entire scope of the claims, can Examiner's enablement rejection be proper?
6. Do Examiner's previous inconsistent statements preclude rejection under § 112?

Claim Rejections - 35 U.S.C. § 103
(claims 1,14-21, 23, 24 and 26-29)

Claim Rejections - 35 U.S.C. § 103
(claims 22 and 25)

7. Does Examiner incorrectly view Applicant's invention as directed to anti-HIV therapy when Applicant's invention is actually directed to increasing systemic tryptophan?
8. Can an obvious rejection be predicated on a non-enabling disclosure?
9. Can an obvious rejection be predicated on what is not known at the time an invention is made, even if the inherency of a certain feature is later established?
10. Given that a positive clinical outcome from high doses of niacin does not necessarily flow from the Examiner cited prior art, can Examiner's obviousness rejection be proper?
11. Should Examiner have provided a basis in fact and/or technical reasoning to reasonably support the determination an increase in systemic tryptophan necessarily flows from the teachings of Tang et. al and Murray et. al.
12. Is Examiner's view of the inherency doctrine overbroad?
13. Can Examiner maintain an unsupported obvious rejection in light of the evidence rebutting

obviousness, including:

- (a) Skepticism of experts
- (b) Long felt need for treating systemic tryptophan depletion in patients infected with a retrovirus.

14. Can Examiner maintain a *prima facie* obvious rejection when Examiner claims the prior art relied upon is unpredictable?

GROUPING OF CLAIMS (37 C.F.R. 1.192(C)(7))

Claims 1, 14-23, and 25-33 do not stand or fall together for every issue. As set forth in section 1 below, claims directed to administering 3 grams of niacin per day to increase plasma tryptophan levels have been expressly enabled by the examples presented in the specification. Similarly, claims directed to administering a dose greater than 100mgs of niacin per day was also disclosed and enabled in the originally filed application. *See*, specification at 8, lines 10-11, and original claim 13. As a result, claims 14, 15, 28, 30 – 33, do not stand or fall together with the rest of the claims for enablement purposes.

ARGUMENT (37 C.F.R. 1.192(C)(8))

I. BACKGROUND

Four HIV infected persons participated in a trial of niacin in the form of nicotinamide. (Specification, p. 9). The participants were at various stages of their HIV infection as judged by their CD4 counts which ranged from 0 to 620. (Specification, Table 1). The participants were receiving either a stable regimen of anti-viral drugs [i.e. anti-HIV drugs] for a period greater than

one year or were not taking any anti-viral drugs. (Specification, p. 9). Two of the participants had known co-infections infections typical of HIV infected persons. *Id.* Each participant took 3 grams of nicotinamide per day for 2 months. *Id.* This treatment was not associated with any adverse side effects. *Id.* Each participant's plasma tryptophan was measured prior to treatment and at the end of treatment. (Specification, Table 3). The average increase of plasma tryptophan of all participants was 43.9%. (Specification, p. 9). This change in tryptophan concentration was statistically significant with a calculated p value of $p=0.0112$ [using paired t-test]. *Id.* The study also measured 4 other plasma amino acids. (Specification, Table 4). All amino acid concentrations were measured by High Performance Liquid Chromotography [HPLC]. (Specification, p. 9). There was no significant change in the plasma amino acid concentrations other than tryptophan. *Id.* Plasma tryptophan changed in a statistically significant manner. (Specification, Tables 3 & 4).

II. ISSUES ON APPEAL

1. **The working examples of the specification preclude a finding of non-enablement--at least for the examples shown.**

"A single working example in the specification for a claimed invention is enough to preclude a rejection which states that nothing is enabled since at least that embodiment is enabled." MPEP § 2164.02. Here, Examiner objects to the specification and has rejected all claims as "failing to adequately teach how to make and/or use the invention, and thereby failing to provide an enabling disclosure." (Paper 12 at 2.) The MPEP, however, expressly precludes such a rejection when at least one example is provided in the specification. MPEP § 2164.02.

In this case, Applicant has provided four working examples. (See Specification, including

Tables 1-4. As expressly set forth in the specification:

Four HIV infected persons participated in a trial of niacin in the form of nicotinamide. The participants were at various stages of their HIV infection as judged by their CD4 counts which ranged from 0 to 620 [see table 1]. The participants were receiving either a stable regimen of anti-viral drugs [i.e. anti-HIV drugs] for a period greater than one year or were not taking any anti-viral drugs. Two of the participants had known co-infections typical of HIV infected persons. Each participant took 3 grams of nicotinamide per day for 2 months. This treatment was not associated with any adverse side effects. Each participant's plasma tryptophan was measured prior to treatment and at the end of treatment [see table 3]. The average increase of plasma tryptophan of all participants was 43.9%. This change in tryptophan concentration was statistically significant with a calculated p value of $p=0.0112$ [using paired t-test]. The study also measured 4 other plasma amino acids which are listed in table 4. All amino acid concentrations were measured by High Performance Liquid Chromatography [HPLC]. There was no significant change in the plasma amino acid concentrations other than tryptophan. As demonstrated in tables 3 and 4, only plasma tryptophan changed in a statistically significant manner.

(emphasis supplied). Thus, at a minimum, claims directed to administering 3 grams of niacin per day to increase plasma tryptophan levels have been enabled by the examples presented in the specification.

2. The Federal Circuit has expressly rejected the notion that controlled testing in a limited number of humans is insufficient.

The MPEP notes that examiners should give "special consideration" to asserted therapeutic or pharmacological utilities. See MPEP § 2107.03. While Examiner's rejection is nominally based on non-enablement under §112 not lack of utility under §101, Examiner's §112 rejection effectively and improperly requires Applicant to have completed Phase II testing. Effectively requiring Application to have completed Phase II testing is improper.

In an opinion quite critical of a §112 (first paragraph) rejection, the Federal Circuit warned

against rejecting a claim under §112 because testing had only been performed on a limited number of individuals and limited dosage regimes. *See, e.g., In Re Brana*, 51 F.2d 1560 ¶ 35 (Fed. Cir. 1995). In *Brana*, the Court described the difference between Phase I and Phase II testing. Phase I testing is typically based on limited human studies or animal studies. Phase II testing, on the other hand, is more extensive and often used to “determine primarily...[a drug’s] potential efficacy under different dosage regimes” and to apply the dosage regimes to a large sample of humans. *Id.*

As it had on several occasions before, the Court denounced the notion that a patent applicant needs to provide a level of detail that could only be achieved through Phase II testing. Indeed, the Court expressly stated “[w]ere we to require phase II testing in order to prove utility, the associated costs would prevent many companies from obtaining patent protection on promising new inventions, thereby eliminating an incentive to pursue, through research and development, potential cures in many crucial areas such as the treatment of cancer.” *Id.* at ¶ 36. As a result, the court concluded that the applicant’s disclosure complied with 35 U.S.C. § 112.

Here, Applicant has not conducted Phase II testing. However, Examiner’s basis of rejection under §112 effectively requires applicant to have completed Phase II testing. Examiner’s basis is too stringent. Applicant disclosed all of its Phase I testing in its application. The Phase I testing showed a statistically significant change in systemic tryptophan based on administration of niacin compounds in patients with normal dietary intakes of niacin and tryptophan. No one had ever discovered this use for niacin before.

Examiner’s rejection that Applicant has “failed to provide sufficient working examples” cannot be reconciled with *In Re Brana*. In order for Applicant to provide more examples in his specification, Applicant would have had to conduct Phase II testing. Thus, for Examiner to

criticize Applicant for not providing more examples is improper. Similarly, Examiner's rejection that "these examples are not exhaustive, nor define a class of compounds" is equally improper. As noted by the *Brana* court, Phase II testing is used to "determine primarily...[a drug's] potential efficacy under different dosage regimes" and to apply the dosage regimes to a large sample of humans. *Id.* For the Examiner to require "exhaustive examples" that "define a class of compounds" improperly requires Applicant to have completed Phase II testing.

3. By failing to provide support for why one would not expect to be able to extrapolate the four examples over the entire scope of the claims, Examiner's rejection is improper.

"To make a valid rejection, one must evaluate all the facts and evidence and state why one would not expect to be able to extrapolate that one example across the entire scope of the claims." MPEP § 2164.02. Here, Examiner baldly asserts that the "pharmaceutical art is unpredictable" but does not explain why one would not expect to be able to extrapolate the four examples over the entire scope of the claims. Applicant offers controverting evidence that one skilled in the art would expect to be able to extrapolate the examples provided in the specification across the entire scope of the claims. Sideb'm Decl., ¶ 14; Mur. Decl., ¶ 33-34.

The application provides statistically significant human clinical data with respect to the administration of 3 gms/day of niacin. *See* Specification, Table 3. It is known in the art that a normal dietary intake of niacin by one infected with a retrovirus can still leads to systemic tryptophan depletion in some cases. *See* Mur. Decl., ¶ 20; Exhibit B to Mur. Decl. One

knowledgeable in the field of infectious diseases could reasonably extrapolate the findings at 3 gms/day to a range in excess of a normal dietary intake of niacin. Sideb'm Decl., ¶ 14; Mur. Decl., ¶ 33-34. Given that the recommended daily dose of niacin is approximately 20 milligrams per day, one would expect a favorable systemic tryptophan result between a range of 100 mgs/day and higher. Sideb'm Decl., ¶ 14; Mur. Decl., ¶ 33-34.

4. The *Wands*' factors favor enablement.

Pursuant to *In Re Wands* and MPEP § 2164.01(a), the following factors are relevant to the issue of enablement: (a) the quantity of experimentation needed to make or use the invention based on the content of the disclosure, (b) the amount of direction provided by the inventor, (c) the existence of working examples, (d) the nature of the invention, (e) the state of the prior art, (f) the relative skill of those in the art (g); the level of predictability in the art, and (f) the breadth of the claims. If a patent application would have taught one skilled in the art how to use the full scope of the claimed invention without undue experimentation, such application has been enabled. *See, In re Wright*, 999 F.2d 1557,1562, 27 USPQ2d 1510, 1513 (Fed. Cir. 1993).

In this case, as set forth in more detail in the following sections, Examiner's rejection under § 112 (first paragraph) was improper given that: (a) no experimentation is needed to make or use the invention based on the content of the disclosure, (b) the specification contains within it a connotation of how to use the invention, (c) the direction provided by the inventor enables one skilled in the art to practice the invention, (d) Examiner's requiring "exhaustive examples" that "define a class of compounds" is improper after the Federal Circuit has expressly rejected the notion that controlled testing in a limited number of humans is insufficient, (e) the state of the prior

art favors enablement, (f) the relative skill of those in the art favors enablement -- the art recognizes that standard modes of administration are known and contemplated, and (g) the breadth of the claims favor enablement?

a. No experimentation is needed to make or use the invention based on the content of the disclosure.

“A patent need not teach, and preferably omits, what is well known in the art.” MPEP § 2164.01, citing *In re Buchner*, 929 F.2d 660, 661, 18 USPQ2d 1331, 1332 (Fed. Cir. 1991); *Hybritech, Inc. v. Monoclonal Antibodies, Inc.*, 802 F.2d 1367, 1384, 231 USPQ 81, 94 (Fed. Cir. 1986), cert. denied, 480 U.S. 947 (1987). “The test of enablement is not whether any experimentation is necessary, but whether, if experimentation is necessary, it is undue.” *In re Angstadt*, 537 F.2d 498, 504, 190 USPQ 214, 219 (CCPA 1976).

In this case, the patent application enables a person who regularly treated patients with retroviruses to use the invention claimed. *See generally*, declarations of David Sidebottom, MD, (“Sideb’m Decl.”) and Michael F. Murray, MD (“Mur. Decl.”). For one thing, even though the specification need not disclose what is well known to those skilled in the art, the application provides information on the administration of niacin and well as cites references for the reader to learn more information about the administration and effects of niacin. *See generally*, application; *see also*, Sideb’m Decl., ¶ 8(a); Mur. Decl., ¶ 27(a). In addition, medical doctors or those in the medical field are often familiar with niacin. Sideb’m Decl., ¶ 8(b); Mur. Decl., ¶ 27(a). Those who are not readily familiar with niacin know that much information can be found regarding the administration and effects of niacin on “Medline”, in journals, books and other commonly available resources. *Id.*

The application tells a reader that the preferred method to combat plasma tryptophan depletion is to “administer niacin in `pharmacological doses””. Sideb’m Decl., ¶ 8(c); Mur. Decl., ¶ 27(b); *see also* application, pg. 7, line 12. The application recommends that a reader administer a dose greater than 20 milligrams per day because a lesser dose would not be expected to produce the pharmacological effect of combating plasma tryptophan depletion. Sideb’m Decl., ¶ 8(d); Mur. Decl., ¶ 27(c); *see also* application, pg 8, line 1.

The application informs a reader that the preferred dose is 500 milligrams of niacin per day. Sideb’m Decl., ¶ 8(h); Mur. Decl., ¶ 27(g); *see also* application, pg. 9, lines 3-4. The application informs a reader to expect pharmacological activity to begin occurring at a dose of 100 milligrams per day. Sideb’m Decl., ¶ 8(e); Mur. Decl., ¶ 27(d); *see also* application, pg. 8, lines 10-11. The application informs a reader to expect a patient to undergo a reverse systemic tryptophan depletion upon the daily administration of 100 milligrams of niacin. Sideb’m Decl., ¶ 8(f); Mur. Decl., ¶ 27(e); *see also* application, pg. 8, lines 10-15. The application informs a reader that the preferred method of administration of niacin in this invention is oral administration. Sideb’m Decl., ¶ 8(g); Mur. Decl., ¶ 27(f); *see also* application, pg 9, lines 2-3. The application informs a reader that the preferred form of niacin to practice this invention is nicotinamide. Sideb’m Decl., ¶ 8(i); Mur. Decl., ¶ 27(h); *see also* application, pg. 9, lines 3-4.

By way of example, the application informs a reader that administering 3 grams of nicotinamide per day for two months can be expected to increase plasma tryptophan between 20% and 80%. Sideb’m Decl., ¶ 8(j); Mur. Decl., ¶ 27(i); *see also* application, Table 3. It is known that a safe maximum dose for nicotinamide is 3 grams per day and this readily supported by the medical literature. Mur. Decl., ¶ 27(j); M. Knip et al., Safety of High-Dose Nicotinamide: A

Review, 43 Diabetologia 1337-1345 (2000), a copy of which is attached to the Mur. Decl. as Exhibit E.

The laboratory parameter to monitor plasma tryptophan concentrations is widely available. Mur. Decl., ¶ 27(k). The application provides expected baseline systemic tryptophan levels and expected increases in systemic tryptophan. Mur. Decl., ¶ 27(l); *see also* application, Table 3. Furthermore, medical literature provides gives expected and target tryptophan levels. Mur. Decl., ¶ 27(l). For example, Werner et al came up with 91 micromol/l as a baseline for systemic tryptophan from their study on tryptophan and HIV in 1988. *See Exhibit B* to Mur. Decl.. Other studies have come up with different normal tryptophan levels - generally lower than 91 - but Werner and colleagues have stated their normal as 91 in three different studies. *See id.* Medical literature also establishes goal levels for tryptophan [by comparing tryptophan values in patients with HIV infection to healthy control patients] make this an easily administered pharmacological agent. *Id.*

For all of the foregoing reasons, no undue experimentation is necessary before someone in the medical field could practice this invention.

b. The specification contains within it a connotation of how to use the invention.

“35 U.S.C. 112 is satisfied if a statement of utility in the specification contains within it a connotation of how to use the invention....” MPEP § 2164.01(c). “For example, it is not necessary to specify the dosage or method of use if it is known to one skilled in the art that such information could be obtained without undue experimentation. If one skilled in the art, based on knowledge of compounds having similar physiological or biological activity, would be able to discern an appropriate dosage or method of use without undue experimentation, this would be sufficient to

satisfy 35 U.S.C. 112, first paragraph.” *Id.*; see, e.g., *Application of Johnson*, 282 F.2d 370 (P.App. Cir. 1960).

In this case, it is typical that patients with retroviral infections will periodically ask their physician to administer a “non-prescription” therapy, and physicians will then work with patients in an attempt to safely achieve the trial of therapy requested. Mur. Decl., ¶ 26(a). In fact, in a recent study by Hsiao et al. over half of the retrovirally infected patients in the study were taking non-prescription therapies, and two-thirds of those patients discussed these therapies with their physicians. *See id.*

In addition, those in the medical field recognize that no two patients are the same. Sideb’m Decl., ¶ 9; Mur. Decl., ¶ 28. Those in the medical field also recognize that different patients react differently to the same treatment. *Id.* Patients react differently to the same treatment for a myriad of reasons including different diets, different stress levels, different genetic makeup, and different metabolic rates. *Id.* These observations make the determination of optimal dosing of a drug in a particular case an individualized process. *Id.* Against this backdrop, the specification enables one in the medical field to practice the invention without undue experimentation. *See* Sideb’m Decl., ¶ 11-13; Mur. Decl., ¶ 30(a).

Based on the information contained in the application, a doctor would likely, in the ordinary case, confirm both retroviral infection and tryptophan depletion with simple blood tests, and then initiate treatment for tryptophan depletion by orally administering a daily dose of nicotinamide in the preferred amount of 500 milligrams per day. Sideb’m Decl., ¶ 10(a); Mur. Decl., ¶ 29(a). In a more extreme case of tryptophan depletion, a doctor would likely initiate treatment by orally administering a daily dose of nicotinamide in the preferred amount of 3 grams per day. Sideb’m

Decl., ¶ 10(b); Mur. Decl., ¶ 29(b). In either case, a doctor would probably re-assess the patient at a subsequent date. Sideb'm Decl., ¶ 10(c); Mur. Decl., ¶ 29(c). If the tryptophan levels had increased a doctor would maintain the treatment until tryptophan level had returned to an appropriate level. Sideb'm Decl., ¶ 10(c); Mur. Decl., ¶ 29(c). If the tryptophan level had not increased, a doctor would raise the dosage commensurate with the condition. Sideb'm Decl., ¶ 10(c); Mur. Decl., ¶ 29(c).

Thus, no undue experimentation is necessary for someone in the medical field to practice this invention.

c. The direction provided by the inventor enables one skilled in the art to practice the invention.

"[A]n extended period of experimentation may not be undue if the skilled artisan is given sufficient direction or guidance." MPEP § 2164.06 citing *In re Colianni*, 561 F.2d 220, 224, 195 USPQ 150, 153 (CCPA 1977). " 'The test is not merely quantitative, since a considerable amount of experimentation is permissible, if it is merely routine, or if the specification in question provides a reasonable amount of guidance with respect to the direction in which the experimentation should proceed.' " MPEP § 2164.06, quoting *In re Wands*, 858 F.2d 731, 737, 8 USPQ2d 1400, 1404 (Fed. Cir. 1988) (citing *In re Angstadt*, 537 F.2d 489, 502-04, 190 USPQ 214, 217-19 (CCPA 1976)).

For example, in *United States v. Telectronics, Inc.*, 857 F.2d 778, 8 USPQ2d 1217 (Fed. Cir. 1988), *cert. denied*, 490 U.S. 1046 (1989), the court reversed the findings of the district court for lack of clear and convincing proof that undue experimentation was needed. *Id.*; *see also* MPEP § 2164.06. The *Techtronics* court ruled that since one embodiment and the method to determine

dose/response was set forth in the specification, the specification was enabling. *Id.* The question of time and expense of such studies, approximately \$50,000 and 6-12 months standing alone, failed to show undue experimentation.” MPEP § 2164.06.

In this case, Applicant has provided evidence that no experimentation is necessary to practice the invention. *See generally*, Sidebottom and Murray declarations and argument provided in section 4(a), *supra*, pages 12-14.

d. The state of the prior art favors enablement.

“The state of the prior art is what one skilled in the art would have known, at the time the application was filed, about the subject matter to which the claimed invention pertains.” MPEP § 2164.05(a) “The state of the prior art provides evidence for the degree of predictability in the art and is related to the amount of direction or guidance needed in the specification as filed to meet the enablement requirement.” MPEP § 2164.05(a). “The state of the prior art is also related to the need for working examples in the specification.” MPEP § 2164.05(a). “In general, the pertinent art should be defined in terms of the problem to be solved rather than in terms of the technology area, industry, trade, etc. for which the invention is used.” MPEP § 2164.05(a).

In this case, the state of the prior art with respect to the administration of niacin was well developed at the time the application was filed. *See generally*, Sidebottom and Murray declarations and argument provided in section 4(a), *supra*, pages 12-14. Those in the art knew the various ways to administer niacin as well as the upper limits of safe doses. Mur. Decl., ¶ 27. What was unknown in the art was that the administration of niacin alleviates systemic tryptophan. *Id.*, ¶ 6-20. The application provided this previously unknown aspect. *Id.* Thus, the combination of

what was known in the art and what was provided by the application has enabled those in the art to practice the invention. *See generally*, Sidebottom and Murray declarations and argument provided in section 4(a), *supra*, pages 12-14.

e. The relative skill of those in the art favors enablement -- the art recognizes that standard modes of administration are known and contemplated.

“35 U.S.C. 112 is satisfied if ... the art recognizes that standard modes of administration are known and contemplated.” MPEP § 2164.01(c). “If multiple uses for claimed compounds or compositions are disclosed in the application, then an enablement rejection must include an explanation, sufficiently supported by the evidence, why the specification fails to enable each disclosed use. In other words, if any use is enabled when multiple uses are disclosed, the application is enabling for the claimed invention.” *Id.*

Applicant claims a “method of increasing systemic tryptophan comprising the administration of an effective amount of niacin for increasing systemic tryptophan...” (See Claim 1). An effective amount begins at a “pharmacological dose” of niacin. (Specification, p. 7, ll. 13-17). Prior to June 30, 2000 it was known that a pharmacological dose is a dose “whereby a pharmacodynamic action is evidenced that is distinct from the nutrient function.” See e.g., Joseph R. DiPalma and William S. Thayer, Use of Niacin as a Drug, 11 Annu. Rev. Nutr. 169-87, 170 (1991), which was cited on page 7 of the Specification. In this case, a pharmacological dose is the dose at which niacin stops acting as a nutrient and starts acting to inhibit and/or reverse the retroviral induced metabolic derangement. (Specification, p. 7-8).

The Specification disclosed “A pharmacological dose of niacin generally occurs at a dose of about 100 milligrams per day....” (Specification, p. 8, line 10). Reading the “description”

section of the application as a whole, the 100 mg/day dose is the lower limit of pharmacologic activity. *Id.* As shown in by the four examples set forth in the specification, 3 g/day is also an effective pharmacologic dose. (Specification, p, 9-10). 500 mg/day is the preferred pharmacologic dose. (Specification, p. 9, line 3). Thus, applicant has defined the lower limit of the metes and bounds of the claimed invention as 100 mg/day.

The upper limit was also known in the art prior to June 30, 2000. The safety of high doses of niacin in the form of nicotinamide and nicotinic acid has been explored by those in the art for more than 50 years. Mur. Decl., ¶ 27(j); *see also*, M. Knip et al., Safety of High-Dose Nicotinamide: A Review, 43 Diabetologia 1337-1345 (2000) which is attached to Murray's Declaration at Exhibit E. It was known prior to June 30, 2000 that doses up to 3 g/day are considered safe and well tolerated. *Id.* at 1343-44. Higher doses of up to 6 g/day have exhibited some side effects, including nausea. *Id.* at 1343. And, a case of reversible hepatotoxicity occurred in a patient taking 9 g/day. *Id.* No other significant side effects were reported.

Applicant has claimed a specific use for niacin in doses that are greater than 100 mg/day and less than the toxic limit, which is believed to be in excess of 9g/day. At a minimum, applicant has enabled a range of pharmacologic doses between 100 mg/day and 9 g/day. Accordingly, Applicant respectfully requests that the Board find that the instant claims do not "necessitate an exhaustive search for the embodiments suitable to practice the claimed invention" and that the instant claims do not fail to specify the "metes and bounds" of the patent protection desired.

f. The breadth of the claims favor enablement

"Where the invention resides in finding the activity rather than in discovering some critical range or the like, we have approved of such broad definitions of quantity or dosage. *In re*

Caldwell, 319 F.2d 254, 50 CCPA 1464 (1963); compare *In re Halleck*, 422 F.2d 911, 57 CCPA (1970); *Application of Gardner*, 427 F.2d 786, 788 (P.App. Cir. 1970) (“Claims 4 and 5 call for “daily dosages” in the ranges 10 to 450 mg. and 10 to 300 mg., respectively. They are enormously wide ranges but there is nothing indefinite about them”).

In this case, the invention centers on the discovery that the administration of a daily dose of niacin in patients already receiving adequate dietary intakes of both tryptophan and niacin results in increased levels of systemic tryptophan. No one had ever considered this before. *See* Mur. Decl., ¶¶ 6-20. Thus, broad claims to this invention are appropriate.

- 5. By disclosing at least one method for making and using the claimed invention that bears a reasonable correlation to the entire scope of the claims, Examiner’s § 112 rejection is improper.**

“The enablement requirement of 35 U.S.C. § 112 is satisfied as long as the specification discloses at least one method for making and using the claimed invention that bears a reasonable correlation to the entire scope of the claim.” MPEP § 2164.01(b). As previously noted, Applicant has provided four working examples. Thus, at a minimum, claims directed to administering 3 grams of niacin per day to increase plasma tryptophan levels have been enabled by the examples presented in the specification.

Furthermore, Pages 7 and 8 of Applicant’s Specification describe what the term “pharmacological dose” means. The balance of the application sets forth the “metes and bounds.” As stated on page 8, line 10 of the Applicant’s application, “a pharmacological dose of niacin generally occurs at a dose of about 100 milligrams per day....” Reading the “description” section of the application as a whole, the 100 mg/day dose is the lower limit of pharmacologic activity. As

shown in by the four examples set forth in the application, 3 g/day is also an effective pharmacologic dose. And, as stated on page 9, line 3, 500 mg/day is the preferred pharmacologic dose. Thus, applicant has defined the lower limit of the metes and bounds of the claimed invention as 100 mg/day.

Applicant has claimed a specific use for niacin in an “effective amount.” As disclosed and enabled in the application an effective amount is one that creates pharmacologic activity. It is expected that an effective amount would begin to occur at a dose greater than 100 mg/day. At a minimum, applicant has enabled a range of pharmacologic doses between 100 mg/day (the lower limit) and 3 g/day (the dose administered in the four examples). Accordingly, Applicant respectfully requests that the Board conclude that the instant claims do not “necessitate an exhaustive search for the embodiments suitable to practice the claimed invention” and that the instant claims do not fail to specify the “metes and bounds” of the patent protection desired.

6. Examiner’s rejection under § 112 is inconsistent with earlier positions taken by Examiner.

The Examiner has rejected all pending claims under 35 U.S.C. § 112, second paragraph, as “indefinite” and “failing to set forth the metes and bounds of the patent protection desired.” Further, Examiner claims that an “effective amount” is not set forth in the specification. Applicant has previously demonstrated that the application enabled the metes and bounds of the claims. *See* Brief, *supra*.

In light of the Examiner’s previous suggestion on page 10 (paper 7) to use a different word than “pharmacological”, Applicant has since substituted the word “effective amount” for the

recited “pharmacological” in the relevant claims. The use of the term “effective amount” has been held appropriate in similar cases. *See, In re Caldwell*, 319 F.2d 254, 50 CCPA 1464 (1963) (“Where the invention resides in finding the activity rather than in discovering some critical range or the like, we have approved of such broad definitions of quantity or dosage”); *In re Halleck*, 442 F.2d 911 (C.C.P.A. 1970) (approving use of the term “effective amount”); *Application of Gardner*, 427 F.2d 786, 788 (P.App. Cir. 1970) (“Claims 4 and 5 call for “daily dosages” in the ranges 10 to 450 mg. and 10 to 300 mg., respectively. They are enormously wide ranges but there is nothing indefinite about them.”). Furthermore, the term “effective amount” is expressly used in the specification (page 5, line 4-5 and page 6, line 11) and is unmistakably implied by the specification as a whole. Thus, Examiner’s rejection is improper.

Claim Rejections - 35 U.S.C. § 103
(claims 1,14-21, 23, 24 and 26-29)

Claim Rejections - 35 U.S.C. § 103
(claims 22 and 25)

Examiner has rejected claims 1, 14-21,23,24 and 26-29 under 35 U.S.C. § 103 as being unpatentable over Tang et. al, Brown et al, in view of Murray et. al.

7. Applicant’s invention is directed to increasing systemic tryptophan not anti-HIV therapy.

Examiner’s states that Tang et al and Brown et al teach that elevated levels of niacin “significantly decreas[es] the progressions of HIV infected individuals to AIDS.” (page 5 of paper 12.) Applicant’s invention is different. The invention set forth in the application is not directed to

antiviral therapy. Specification, p. 6, lines 11-14; *see also* Mur. Decl., ¶¶ 31-32; *see also* Sideb'm Decl., ¶ 13. The invention set forth in the application is directed to treating patients with systemic tryptophan depletion. *Id.*

Moreover, any study claiming niacin for anti-viral effect may not be relied upon due to potential inoperability. The *in vivo* study set forth in the application did not show that the administration of niacin had any anti-viral effect. Mur. Decl., ¶32. No *in vivo* study has been able to show that niacin in any amount has any anti-viral effect. *Id.*

In this case, Applicant has discovered a method of increasing systemic tryptophan of humans through the administration of niacin. Mur. Decl., ¶6. Applicant's invention is new, useful and non-obvious.

8. Obviousness cannot be predicated on a non-enabling disclosure.

Obviousness cannot be predicated on a non-enabling disclosure. MPEP § 2121.01. "A reference contains an 'enabling disclosure' if the public was in possession of the claimed invention before the date of invention. *Id.* "Such possession is effected if one of ordinary skill in the art could have combined the publication's description of the invention with his [or her] own knowledge to make the claimed invention." *In re Donohue*, 766 F.2d 531, 226 USPQ 2141.03

In this case, the public was not in possession of the claimed invention. In late 1998 – after Tang, Brown and Murray 1995 – experts noted that niacin use in treating retroviruses had never been shown and was purely speculative. For example, one expert stated:

many HIV infected people take vitamin supplements and there have never been reports on any major effect on the clinical course except for vitamin A. Many individuals and researchers have ideas that vitamins may provide some clinical benefit in HIV infection by the niacin theory needs

much better substantiation and of course some kind of clinical trial. Thus at this point the concept is so purely speculative...

See, Mur. Decl., ¶¶ 13-20; *See also*, The Lancet peer review attached as Exhibit D to Mur. Decl.

Another expert agreed. *See*, Mur. Decl., ¶ 18; *See also*, The Lancet peer review attached as Exhibit D to Mur. Decl. The other expert also noted that while Tang, et al. claimed a positive clinical outcome with niacin, Tang's study may have been due to "confounding factors" and/or the presence of other B vitamins. *Id.*

In addition to experts in the field declaring speculative what Examiner claims is obvious, the Tang study is simply different. Mur. Decl., ¶20. Niacin occurs naturally in the diet as a nutrient, and it is very difficult to obtain a niacin free diet. *Id.* Tang et al's observation that patients with high verses low nutrient amounts of niacin in their diet correlate with different outcomes is distinct from the patent application examples, where the patients were prospectively given a dose of niacin designed specifically to exceed nutrient amounts and to act in a manner which was pharmacodynamically distinct from nutrient amounts of niacin. *Id.* While Tang et al simply observes a correlation between micronutrient intake levels of niacin and then speculates that it may relate to a role for niacin in "immune function" [page 1252], the examples provided in the patent establish the unexpected finding that high doses of niacin given therapeutically to retrovirally infected patients with normal nutrient intakes results in improved tryptophan levels.

RR Brown did not enable the public either. RR Brown taught treating tryptophan depletion with tryptophan – not niacin. Mur. Decl., ¶ 38-40. Thus, in addition, Brown cannot be relied on because he taught away from niacin therapy. In his 1991 paper titled "Implications of Interferon-

Induced Tryptophan Catabolism in Cancer, Auto-Immune Diseases and AIDS”, Dr. RR Brown discussed the implications of tryptophan metabolism to HIV and AIDS. Dr. RR Brown recognized the importance of looking for a way to therapeutically intervene with respect to systemic tryptophan depletion. *Id.* At no time in his 1991 paper -- or anywhere else -- did Dr. Brown suggest tryptophan depletion could be treated with niacin. *Id.*

In his 1991 paper, Dr. Brown hypothesized that decreased tryptophan might lead to decreased niacin (something that Skurnick later disproved). Dr. Brown also suggested treating tryptophan deficiency with tryptophan. *Id.* Dr. Brown did not suggest niacin therapy for patients with HIV or other retroviral infections. *Id.* Dr. Brown’s failure to suggest niacin cannot be considered an oversight since Dr. Brown is a prominent tryptophan researcher with a body of work encompassing over 100 articles stretching back to the 1950s. *Id.*

Thus, Tang and Brown had not enabled experts in the field to be in possession of niacin therapy for tryptophan depletion as of June 30, 2000. Murray 1995 cannot be relied on either because it is equally non-enabling -- Murray 1995 has already been declared non-enabling by the USPTO. *See* Mur. Decl., ¶¶ 35-37. In 1995, Murray published two articles, both already made of record: (1) MF Murray, et al., Nicotinamide Inhibits HIV-1 in Both Acute and Chronic *In Vitro* Infection, Biochemical and Biophysical Research Communications, 210:954-959 (1995) and (2) MF Murray, et al., HIV Infection Decreases Intracellular Nicotinamide Adenine Dinucleotide [NAD], Biochemical and Biophysical Research Communications, 212:126-131 (1995). (Collectively, these articles are referred to as the “1995 articles.”)

The 1995 articles were based on *in vitro* data. The same *in vitro* data that formed the basis of the 1995 articles also formed the basis of a patent application filed with the United States Patent

and Trademark Office (“USPTO”), U.S. patent application 07/906,689 (the “‘689 application”). A true and correct copy of the ‘689 application is attached to the Mur. Decl. as **Exhibit F**. The USPTO rejected the ‘689 patent application as non-enabling and unpatentable because it lacked *in vivo* substantiation, specifically:

The specifications provide data only for inhibiting HIV in cells in culture. There is no data to substantial the alleged utility for treating human subjects infected with HIV. There is **no data to substantiate the alleged utility for treating human subjects infected with HIV....** Without statistically significant data documenting the claimed method for treating patients, the person of ordinary skill in the art, knowing the unpredictability of extrapolating from in vitro results to in vivo performance, would have good reason to doubt the efficacy of applicant’s invention.

See Exhibit F, USPTO office action, page 2-3, rejections under §101 and § 112 (emphasis supplied). A true and correct copy of the August 25, 1992 office action is also attached to the Mur. Decl. as **Exhibit F**.

The infectious disease community has been aware of the possibility of tryptophan depletion occurring in patients with HIV since at least as early as 1988. *See, e.g.,* Exhibit B to Mur. Decl. None of the medical literature suggests that the administration of niacin to restores tryptophan levels. *See* Exhibit B to Mur. Decl. No one of ordinary skill in the art could have combined the Examiner cited prior art (Tang, Brown, Murray 1995) with his [or her] own knowledge to make the claimed invention as required before prior art can be considered enabling. The medical community and the USPTO found the Examiner’s prior art (Tang, Brown, and Murray 1995) to be non-enabling. Thus, Tang, Brown, and Murray cannot and should not be relied upon here either as the basis for an obvious rejection.

9. Obviousness cannot be predicated on what is not known at the time an invention is made, even if the inherency of a certain feature is later established

Obviousness cannot be predicated on what is not known at the time an invention is made, even if the inherency of a certain feature is later established. *In re Rijckaert*, 9 F.2d 1531, 28 USPQ2d 1955 (Fed. Cir. 1993). In this case, the infectious disease community has been aware of the possibility of tryptophan depletion occurring in patients with HIV since at least as early as 1988. *See, e.g.*, Exhibit B to Mur. Decl. Nowhere has anyone other than Murray 1999, which is not “prior art”, suggested the administration of niacin to restore tryptophan levels. Mur. Decl., ¶¶ 7-8.

10. A positive clinical outcome from high doses of niacin does not necessarily flow from the Examiner cited prior art.

An “inherent characteristic” necessarily flows from the teachings of the prior art.” MPEP § 2112, (Section titled *Examiner Must Provide Rationale of Evidence Tending to Show Inherency*)(citing *Ex Parte Levy*, 17 USPQ2d 1461, 1464 (Bd. Pat. App. & Inter. 1990)(emphasis in original). “The mere fact that a certain thing *may* result from a given set of circumstances is not sufficient [to establish inherency.]” *In Re Rijckaert*, 9 F.3d 1531 (Fed. Cir. 1993)(quoting *In re Oelrich*, 666 F.2d 578, 581-82, 212 USPQ 323, 326 (CCPA 1981). In this case, not all HIV patients have depleted tryptophan levels. Mur. Decl., ¶ 6.

Here, Examiner’s claim that Applicant’s invention is inherent in light of Tang et al because Tang et al allegedly teaches a positive clinical outcome for HIV infected patients is incorrect. While there is a correlation between retroviral infection and tryptophan depletion there is significant interpatient variability. Mur. Decl., ¶ 6; *see also* Exhibit B to Mur. Decl.. Thus, not

every patient infected with a retrovirus will necessarily require the intervention suggested by the invention disclosed in the application. *Id.* For example, a patient infected with a retrovirus but having no tryptophan depletion will not benefit from this invention. The invention disclosed in the application will specifically benefit patients in need of therapy to maintain or increase their systemic tryptophan levels. *Id.* As a result, Examiner's citation to the inherency doctrine is not correct because a positive clinical outcome will not necessarily occur through the practice of this invention on all retrovirally infected patients.

11. Examiner must provide a basis in fact and/or technical reasoning to reasonably support the determination an increase in systemic tryptophan necessarily flows from the teachings of Tang et. al and Murray et. al.

Nothing in Tang et al suggests that niacin increases systemic tryptophan levels.

"In relying upon the theory of inherency, the examiner must provide a basis in fact and/or technical reasoning to reasonably support the determination that the allegedly inherent characteristic necessarily flows from the teachings of the prior art." MPEP § 2112, (Section titled *Examiner Must Provide Rationale of Evidence Tending to Show Inherency*)(citing *Ex Parte Levy*, 17 USPQ2d 1461, 1464 (Bd. Pat. App. & Inter. 1990)(emphasis in original). "The mere fact that a certain thing *may* result from a given set of circumstances is not sufficient [to establish inherency.]" *In Re Rijckaert*, 9 F.3d 1531 (Fed. Cir. 1993)(quoting *In re Oelrich*, 666 F.2d 578, 581-82, 212 USPQ 323, 326 (CCPA 1981).

In this case, the Examiner claims that an increase in systemic tryptophan is an "inherent characteristic" that flows from teachings of Tang and Murray. An increase in systemic tryptophan, however, cannot be said to necessarily flow from the teachings of Tang and Murray. The teachings

of Tang concede that the observational data they gathered did not necessarily support a conclusion that niacin has any effect at all:

Since intakes of B-group vitamins are highly **intercorrelated**, further research is needed to determine if one or more of these nutrients is related to HIV-1 disease progression. Under these circumstances, **niacin may represent a marker** of overall intake of B-group vitamins rather than having any direct effect on immune function.

Tang et. al, at 948 (emphasis supplied). Tang, et. al concede that their findings have, at best, only “some biological plausibility.” Tang et. al, at 948. More importantly, though, nothing in Tang et al suggests that niacin increases systemic tryptophan levels.

Similarly, an increase in systemic tryptophan cannot be said to necessarily flow from the *in vivo* teaching Murray et al. Murray et al evaluated nicotinamide as an inhibitor of HIV *in vitro*.

Finally, while there is a correlation between retroviral infection and tryptophan depletion there is significant interpatient variability. Mur. Decl., ¶ 6. Thus, not every patient infected with a retrovirus will necessarily require the intervention suggested by the invention disclosed in the application. *Id.* The invention disclosed in the application will specifically benefit patients in need of therapy to maintain or increase their systemic tryptophan levels. *Id.*

The Court should overrule Examiner’s rejection because Examiner must provide a basis in fact and/or technical reasoning to reasonably support that increased systemic tryptophan necessarily flows from the teachings of Tang et al and Murray et al as required by MPEP § 2112.

12. Examiner’s view of the inherency doctrine is overbroad.

An “inherent characteristic” necessarily flows from the teachings of the prior art.” MPEP § 2112, (Section titled *Examiner Must Provide Rationale of Evidence Tending to Show*

Inherency)(citing *Ex Parte Levy*, 17 USPQ2d 1461, 1464 (Bd. Pat. App. & Inter. 1990)(emphasis in original). Claims directed to the novel application are not obvious unless they necessarily flow from the prior art. See e.g., *In re Halleck* 422 F.2d 911 (C.C.P.A. 1970); *In re Caldwell*, 50 C.C.P.A. 1464, 1468-69, 319 F.2d 254, 257-58 (1963).

In *Halleck*, a case remarkably similar to this case, the applicant sought method claims directed to “animal feed and an effective amount of a peristalsis-regulating substance contained therein for growth stimulation.” 422 F.2d at 912. The Merck “reference disclosed use of the parasympatholytic agents for relaxing smooth muscles,” in similar amounts as claimed by the applicant. *Id.* at 912. The Examiner denied the claims as inherently obvious because Merck disclosed administration of the same substance in similar amounts as the claim at issue, and was thus “inherently” disclosed by Merck. *Id.* In addition, the examiner rejected the claims as obvious in light of Goodman in view of a Journal Article and Merck because those references suggested an increased caloric intake when intestinal time is increased. *Id.* In light of these references, the Examiner concluded that it would be “obvious to increase intestinal time in order to improve utilization of the feed and growth.” *Id.*

The Court of Customs and Patent Appeals (“CCPA”) rejected both of the examiner’s arguments because:

Appellant’s invention is not merely a composition comprising an animal feed and a peristalsis-regulating substance nor a method of administering a peristalsis-regulating substance to animals. Rather, what is alleged to be novel and unobvious is the discovery that a peristalsis-regulating substance will stimulate animal growth. No prior art suggests this.

Id. (emphasis supplied). The CCPA found that the claims were not obvious because the prior art of record was silent with respect to stimulating animal growth by administration of

peristalsis-regulating substances. *Id.* at 914. In this case, the prior art is silent with respect to increasing systemic tryptophan with the use of niacin in patients with normal dietary intakes of niacin and tryptophan. As such, the claims are not inherently obvious.

In *Caldwell*, another case remarkably similar to this case, the applicant sought method claims directed to “supplying an effective amount of aspirin for growth stimulation.” *Id.*, 50 C.C.P.A. at 1465, 319 F.2d at 255. The Court of Customs and Patent Appeals (“CCPA”) noted that while aspirin had been administered to children and rats and the growth rates measured, the claims were not obvious because nothing in the prior art suggested the use claimed by the applicant:

Although aspirin is practically our national drug, it does not appear from anything on the record that its use as a growth promoter for any animal, human or otherwise, has ever been even suggested. As for the reference, we are in complete agreement with the appellant, whose brief states: ‘It seems pretty clear that the Gross reference stands for, and suggests, only one thing as far as the present case goes. That is, that feeding aspirin to children and rats over prolonged periods does not interfere with or retard growth of these two species of animals. As far as aspirin goes, this is the only teaching that can be derived from the reference.’”

Caldwell, 50 C.C.P.A. at 1466, 319 F.2d at 256. The CCPA went on to say that the “real novelty” is “stimulating the growth of ruminants, poultry, or swine by feeding them aspirin for that purpose.” *Id.* 50 C.C.P.A. at 1468, 319 F.2d at 257 (emphasis supplied). “We therefore disagree...that the “real novelty” must reside in the amount of aspirin fed, rather than in the feeding of aspirin for the stated purpose.” *Id.*

In this case, Examiner claims that Applicants invention is not patentable because it is “inherent” in the prior art.” (See pages 6-7 of paper 12). Examiner’s interpretation of the “inherency” doctrine goes too far. Just like the applicant in both *Halleck* and *Caldwell*, Applicant here claims a novel purpose for a known substance: administering an effective amount of niacin to

increase systemic tryptophan of dietarily replete humans. Mur. Decl., ¶6. Nothing in the prior art suggests as much. *Id.*, ¶¶ 7-8. The mere fact that others have observed that higher nutrient doses of niacin are sometimes taken by patients with HIV, and hypothesize that it may be “biologically plausible”¹ that high doses of niacin could slow the onset of AIDS does not render obvious Applicant’s claim to a wholly different purpose. Applicant respectfully requests that the obvious rejection be overruled.

13. Examiner cannot maintain an obvious rejection in light of the evidence rebutting obviousness.

a. Applicant has provided evidence reflecting skepticism of experts, which rebuts an obviousness rejection.

“Expressions of disbelief by experts constitute strong evidence of nonobviousness.” *Environmental Designs, Ltd. v. Union Oil of Cal.*, 713 F.2d 693 (Fed. Cir. 1983)(citing *United States v. Adams*, 383 U.S. 39, 52, 148 USPQ 479, 483-484 (1966)) (The patented process converted all the sulfur compounds in a certain effluent gas stream to hydrogen sulfide, and thereafter treated the resulting effluent for removal of hydrogen sulfide. Before learning of the patented process, chemical experts, aware of earlier failed efforts to reduce the sulfur content of effluent gas streams, were of the opinion that reducing sulfur compounds to hydrogen sulfide would not adequately solve the problem.); *see also*, MPEP§ 716.05

”The skepticism of an expert, expressed before these inventors proved him wrong, is entitled to fair evidentiary weight, . . . as are the five to six years of research that preceded the

¹ See, Tang et. al, at 948 (claiming theory is at best “biologically plausible”).

claimed invention.” *In re Dow Chemical Co.*, 837 F.2d 469, 5 USPQ2d 1529 (Fed. Cir. 1988); *Burlington Industries Inc. v. Quigg*, 822 F.2d 1581, 3 USPQ2d 1436 (Fed. Cir. 1987) (testimony that the invention met with initial incredulity and skepticism of experts was sufficient to rebut the prima facie case of obviousness based on the prior art).

In this case, prior to the filing of the patent application on June 30, 2000, experts in the field of infectious diseases scoffed at the notion that niacin might have some benefit in the treatment of retroviral infections. Mur. Decl., ¶ 13. In late 1998, experts in the field expressed skepticism:

many HIV infected people take vitamin supplements and there have never been reports on any major effect on the clinical course except for vitamin A. Many individuals and researchers have ideas that vitamins may provide some clinical benefit in HIV infection but the niacin theory needs much better substantiation and of course some kind of clinical trial. Thus at this point the concept is so purely speculative...

See, Lancet Peer Review, which is attached as Exhibit D to Murray Decl. (emphasis supplied); *See also* Mur. Decl., ¶¶ 13-20.

Another expert was similarly skeptical. *See*, Lancet Peer Review, which is attached as Exhibit D to Murray Decl. (emphasis supplied); *See also* Mur. Decl., ¶¶ 13-20. The Lancet expert also noted that while Tang, et al. claimed a positive clinical outcome with niacin, Tang’s study may have been due to “confounding factors” and/or the presence of B vitamins. *Id.*

The USPTO was similarly skeptical the information relied on by Murray 1995. *See* Response, *supra*, Section 3, pages 24-25. Murray 1995 articles was based on *in vitro* data. *Id.* The USPTO was skeptical of the *in vivo* data, specifically:

The specifications provide data only for inhibiting HIV in cells in culture. There is no data to substantial the alleged utility for treating human subjects infected with HIV. There is **no data to substantiate the alleged utility for treating human subjects infected with HIV....** Without

statistically significant data documenting the claimed method for treating patients, the person of ordinary skill in the art, knowing the unpredictability of extrapolating from in vitro results to in vivo performance, would have good reason to doubt the efficacy of applicant's invention.

See Mur. Decl., **Exhibit F**, USPTO office action, page 2-3, rejections under §101 and § 112 (emphasis supplied).

Murray's 1999 "niacin hypothesis" cited to Tang and Murray 1995 yet experts in the field scoffed at the "niacin hypothesis" -- very same papers cited by Examiner (Tang, Murray 1995) in support of the obvious rejection. As noted by the Federal Circuit, the skepticism of these experts prior to the filing of the application "constitute[s] strong evidence of nonobviousness." *Environmental Designs, Ltd. v. Union Oil of Cal.*, 713 F.2d 693 (Fed. Cir. 1983); (citing *United States v. Adams*, 383 U.S. 39 (1966)).

b. Applicant has provided evidence reflecting long felt need for treating systemic tryptophan depletion in patients infected with a retrovirus.

Establishing a "long-felt need" for the invention rebuts a finding of obviousness. *See* MPEP § 716.04; § 2141. The infectious disease community has noted, evaluated and discussed that tryptophan depletion can be associated with patients infected with a retrovirus for at least the last sixteen years. Mur. Decl., ¶ 7. For example, attached to the Mur. Decl. as **Exhibit B** are eight articles that examine various aspects of plasma or serum tryptophan in HIV infected patients (referred to as the "eight articles"). Also included as part of **Exhibit B** is a summary prepared by Dr. Murray. The eight articles are not exhaustive of all articles published, but examples of the types of articles that can be found in published medical literature.

The infectious disease community has been aware of the possibility of tryptophan depletion occurring in patients with HIV since at least as early as 1988. *See, e.g.,* Mur. Decl.; **Exhibit B**. The articles reflect the long-felt need for treating tryptophan depletion. *See id.* Nowhere, however, do the eight articles suggest the administration of niacin to restore tryptophan levels. *See id.* Similarly, applicant is aware of no articles that have ever suggested the administration of niacin to restore tryptophan levels. Thus, Applicant's invention is not obvious because it satisfies a long-felt, but unsolved need.

14. A prima facie obvious rejection has not been made in this case.

Pursuant to MPEP 706.02(j), an applicant is not required to submit evidence or a substantive response until a *prima facie* case is made. *In Re Rijckaert*, 9 F.3d 1531 (Fed. Cir. 1993) ("In rejecting claims under 35 U.S.C. § 103, the examiner bears the initial burden of presenting a prima facie case of obviousness. Only if that burden is met, does the burden of coming forward with evidence or argument shift to the applicant." (citations omitted)).

In this case, Applicant contends, and still contends, that the Examiner has not set forth a prima facie case of obviousness for the reasons described in Applicant's previous response dated January 16, 2002. "Obviousness cannot be predicated on what is unknown." *In re Rijckaert*, 9 F.3d 1531 (Fed. Cir. 1993). To establish a *prima facie* case of obviousness, "a reasonable likelihood of success must [] be found in the prior art." MPEP 2142, *Establishing a Prima Facie Case of Obviousness*. According to the MPEP, "at least some degree of predictability is required." MPEP 2143.02. In this case, the prior art cited by the examiner provides no degree of predictability. Therefore, no *prima facie* case can be established.

On page 3 of the first Office Action dated 7/27/2001 (paper 4), Examiner stated that “[t]he pharmaceutical art is unpredictable, requiring each embodiment to be individually assessed for physiological activity.” (emphasis supplied). Examiner continues to assert his unpredictability argument to date.

Applicant concedes that the “verbiage of page 3 (of paper 4) and the verbiage of page 3 (of paper 12) was used by the examiner to support a rejection under 35 U.S.C. § 112. However, the fact that the “verbiage” was used in a §112 rejection does mean it is irrelevant with respect to a *prima facie* analysis under § 103. The predictability (or unpredictability) of the relevant art must remain the same under §103 as it does under §112. The relevant art cannot be “unpredictable” when rejecting a claim under §112, while at the same time being predictable enough to establish a *prima facie* case of obviousness under § 103. Unpredictable under §112 means unpredictable under §103. The verbiage of page 3 (of paper 4 and 12) affirmatively states that the relevant art is “unpredictable”—at least with respect to a §112 rejection. Without predictability, the *prima facie* case of obviousness under § 103 fails. *See* MPEP 2143.02.

In addition, the prior art relied upon by the Examiner further reinforces the conclusion that the prior art is unpredictable. Not only did Tang et. al concede that the data they gathered does not necessarily support a conclusion that niacin had any effect at all on immune function, but Tang et. al admit that, as far as they can tell, niacin may be nothing more than a marker of B-group vitamins:

Since intakes of B-group vitamins are highly **intercorrelated**, further research is needed to determine if one or more of these nutrients is related to HIV-1 disease progression. Under these circumstances, **niacin may represent a marker** of overall intake of B-group vitamins **rather than having any direct effect** on immune function.

(Tang et. al, at 948) (emphasis supplied).

In addition, the study conducted by Tang, et al was based on patient responses to questionnaires. Thus, the Tang study was observational not experimental. Tang, et al. expressly highlight several limitations to their study, including that the “food frequency questionnaire” has not been validated in any HIV-1 seropositive population (Tang et. al, at 950). More importantly, though, Tang, et al. admit that dietary changes of the study’s participants may not have any effect at all on disease progression. *Id.* In fact, dietary changes of the participants may be “a result of disease progression, rather than a cause.” *Id.* At best, Tang, et. al merely claim that their “findings appear to have some biological plausibility.” *Id.*

Similarly, the *in vivo* teaching of Murray et al. is similarly unpredictable. Murray et al expressly state that their extrapolation of *in vitro* data was merely speculation and hypothesis that could only be confirmed by *in vivo* data:

We speculate that NAM works to inhibit one or more ADP-ribosylation steps which might otherwise deplete the infected cell of NAD.

...

If our original hypothesis that HIV induces a pellagroid state is correct...

...

... **if confirmed on an *in vivo* level.**

Murray et al, at 958-59 (emphasis supplied).

Thus, not only is the pharmaceutical art unpredictable as a whole, the prior art relied upon by the Examiner expressly concedes that their conclusions were speculation and hypothesis that needed further research and verification before they could be relied upon. Because the prior art fails the degree of predictability required by MPEP § 2143.02 and because—as the Examiner has

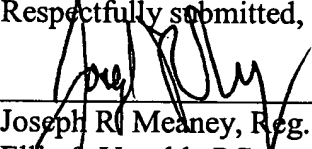
pointed out—the relevant prior art is “unpredictable”, the Examiner cannot establish a *prima facie* case of obviousness. As such, the obviousness rejection must be overruled.

CONCLUSION

For these reasons, Applicant respectfully asserts that it is entitled to a patent as claimed.

Date: September 2, 2004

Respectfully submitted,



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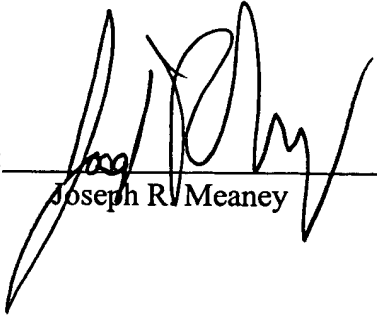
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on

9/3/2004

By:



Joseph R. Meaney

APPENDIX (37 C.F.R. 1.192(c)(9))

The following is a complete listing of all claims:

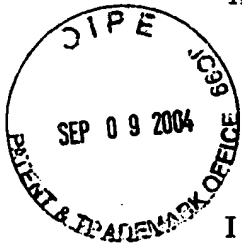
1. (previously amended) A method of increasing systemic tryptophan comprising the administration of an effective amount of niacin for increasing systemic tryptophan to a patient in need of an increase in systemic tryptophan wherein the patient is infected with a retrovirus and wherein the patient has a diet that includes at least the RDA [recommended daily allowance] of niacin and tryptophan.
2. (cancelled)
3. (cancelled)
4. (cancelled)
5. (cancelled)
6. (cancelled)
7. (cancelled)
8. (cancelled)
9. (cancelled)
10. (cancelled)
11. (cancelled)

12. (cancelled)
13. (cancelled)
14. (previously amended) The method of claim 1 wherein the effective amount is greater than 100 milligrams per day.
15. (previously amended) The method of claim 1 wherein the effective amount is approximately 3 grams per day.
16. (previously amended) The method of claim 1 wherein the effective amount exceeds the standard recommended daily amounts for coenzyme activity.
17. (previously amended) The method of claim 1 wherein the effective amount exceeds amounts normally obtainable with routine diet and supplement practices.
18. (previously amended) The method of claim 1 wherein the effective amount exceeds the RDA [recommended daily allowance] of niacin.
19. (previously amended) The method of claim 1 wherein the effective amount is sufficient to raise the intracellular levels of nicotinamide adenine dinucleotide [NAD] in persons with HIV infection.
20. (previously amended) The method of claim 1 wherein the effective amount is sufficient to replete nicotinamide nucleotide precursors [NAD].

21. (previously amended) The method of claim 1 wherein the effective amount of niacin is administered to persons with HIV and other co-infections.
22. (previously amended) The method of claim 1 wherein the effective amount of niacin is administered in combination with antiviral medications selected from the group consisting of reverse transcriptase inhibitors and protease inhibitors.
23. (previously amended) The method of claim 1 wherein the effective amount is administered in combination with other treatments for HIV infection to improve the metabolic status of an infected patient.
24. (cancelled)
25. (previously added) The method of claim 1 wherein the effective amount of niacin is administered in combination with antiviral medications.
26. (previously added) The method of claim 1 wherein the administration occurs by the method selected from the group consisting of oral administration, parenteral administration, rectal administration, pharmaceutical adjuvant administration and pharmaceutical carrier administration.
27. (previously added) The method of claim 1 wherein the niacin is in the form selected from the group consisting of nicotinamide and nicotinic acid.
28. (previously added) The method of claim 1 wherein dietary intake of niacin is less than 100 milligrams.

29. (previously added) The method of claim 1 wherein dietary intake of tryptophan is less than 1.44 grams.
30. (previously added) A method of increasing systemic tryptophan of humans comprising the step of administering an effective amount of niacin to a human patient to increase systemic tryptophan wherein the effective amount of niacin is 100 milligrams per day or greater.
31. (previously added) The method of claim 30 wherein the effective amount is approximately 3 grams per day.
32. (previously added) A method of increasing systemic tryptophan of humans comprising the step of administering an effective amount of niacin to a human patient to increase systemic tryptophan wherein the effective amount of niacin is 100 milligrams per day or greater and wherein the human patient has a plasma tryptophan level of 91 micromol per liter or less.
33. (previously added) The method of claim 32 wherein the effective amount is approximately 3 grams per day.

DECLARATION OF MICHAEL F. MURRAY, M.D.



I hereby declare that:

1. I am the named inventor of United States patent application serial number 09/609,552 (the "552 application"). A copy of the '552 application is attached as **Exhibit A**.

Experience/Expertise of Declarant

2. I am a licensed physician.
3. I am a board certified specialist in infectious diseases.
4. I have been practicing medicine since receiving my medical degree in 1988.
5. I have been treating patients with retroviral infections on a regular basis since 1991.

Applicability of '552 application to patients in need of increase in systemic tryptophan

6. While there is a correlation between retroviral infection and tryptophan depletion there is significant interpatient variability. Thus, not every patient infected with a retrovirus will necessarily require the intervention suggested by the invention disclosed in the '552 application. The invention disclosed in the '552 application will specifically benefit patients in need of therapy to maintain or increase their systemic tryptophan levels.

Long-Felt Need

7. The infectious disease community has noted, evaluated and discussed that tryptophan depletion can often be associated with patients infected with a retrovirus. For example, attached as **Exhibit B** are eight articles that examine various aspects of plasma or serum tryptophan in HIV infected patients (referred to as the "eight articles"). Also included as part of **Exhibit B** is a summary that I prepared of the eight articles. The eight articles of **Exhibit B** are not exhaustive of all articles published, but examples of the types of articles that can be found in published medical literature.
8. The infectious disease community has been aware of the possibility of tryptophan depletion occurring in patients with HIV since at least as early as 1988. See, e.g., **Exhibit B**. Nowhere do the eight articles suggest the administration of niacin to restore tryptophan levels. See **Exhibit B**. Similarly, none of the many articles by other authors that I have

reviewed have ever suggested the administration of niacin to restore tryptophan levels.

Skepticism of Experts in June of 2000

9. At the time the '552 application was filed on June 30, 2000, not only was the infectious disease community not considering the use of niacin to treat tryptophan depletion, the infectious disease community was skeptical of whether niacin had a place at all in the treatment of a patient infected with a retrovirus.
10. For example, I prepared a manuscript titled "Niacin as a Potential AIDS Preventative Factor" (referred to as "my Niacin Hypothesis"), a true and correct copy of which is attached as Exhibit C. My Niacin Hypothesis was ultimately published in the journal *Medical Hypotheses* in November of 1999. *Medical Hypotheses* is a journal that publishes manuscripts on any topic within the broad scope of the biomedical sciences following a limited editorial review, but without expert based peer review.
11. In my Niacin Hypothesis, I hypothesized that HIV infection that therapeutic niacin would act as an AIDS preventative factor. Prior to the filing of the '552 application, there was no published or publicly presented data that supported my hypotheses for treating retrovirally infected patients with niacin or that suggested that niacin therapy had any measurable benefit. In fact, a study by Skurnick et al suggested that retrovirally infected patients had increased levels of niacin in their blood stream, thereby inferring that additional treatment of patients with a compound that they had in excess would be of no obvious value. See Exhibit G, Skurnick, et al. Micronutrient Profiles in HIV-1 Infected Heterosexual Adults, at 80.
12. In a study based on my hypothesis, however, I discovered that the administration of a daily dose of niacin in patients already receiving adequate dietary intakes of both tryptophan and niacin increased levels of systemic tryptophan. See Exhibit A.
13. As demonstrated in the following paragraphs, my Niacin Hypothesis regarding the use of niacin as an AIDS preventative factor was not well received by the infectious disease community prior to the filing of the '552 patent application in June of 2000.
14. Prior to submitting my Niacin Hypothesis to *Medical Hypotheses*, I submitted my Niacin Hypothesis to *The Lancet*. The manuscript that I submitted to *The Lancet* was the same as my Niacin Hypothesis attached hereto as Exhibit C. As described on its website, www.thelancet.com, *The Lancet* is an international general medical journal that will consider any original contribution that advances or illuminates medical science or practice, or that educates the journal's readers.
15. As stated on its web site, before a paper will be published it must be peer reviewed. Although *The Lancet's* web site does not give a detailed description of its review process, it is standard that a manuscript is sent out to 2 experts in the field who comment on the

accuracy, content, and importance of the manuscript.

16. A true and correct copy of *The Lancet's* peer review of my Niacin Hypothesis is attached as **Exhibit D**. *The Lancet* rejection was made in light of Brown 1991 and Tang 1996 - in fact Tang is specifically referenced.

17. One of the peer reviews stated:

many HIV infected people take vitamin supplements and there have never been reports on any major effect on the clinical course except for vitamin A. Many individuals and researchers have ideas that vitamins may provide some clinical benefit in HIV infection but the niacin theory needs much better substantiation and of course some kind of clinical trial. Thus at this point the concept is so purely speculative...

See, Exhibit B, page 2 (emphasis supplied).

18. The other peer review was similarly skeptical. See, Exhibit B, page 3. The other review also noted that while Tang, et al. claimed a positive clinical outcome with niacin, Tang's study may have been due to "confounding factors" and/or the presence of the other B vitamins.
19. I have been practicing in the field of infectious diseases, retroviruses in particular, since 1991. I have kept abreast of medical literature in the field of retroviruses since 1991. When I submitted the '552 application in June of 2000, I was not aware of anyone other than myself who thought that administering niacin to patients with had a retroviral infection and did not have a dietary deficiency of niacin or tryptophan would increase levels of systemic tryptophan in any mammal, including humans. In my opinion, the expert opinions set forth in Exhibit D reflect the opinions of the infectious disease community in June of 2000.
20. It is puzzling to me that experts in the field could declare something speculative that Dr. Travers would declare obvious, but perhaps it has to do with some of the less explicit details which I can outline here. Recall that niacin occurs naturally in the diet as a nutrient, and it is very difficult to obtain a niacin free diet. So when Tang et al observe that patients with high versus low nutrient amounts of niacin in their diet correlate with different outcomes it is viewed scientifically as distinct from when in the '552 application examples [and subsequently in Murray et al 2001], the patients were prospectively given a dose of niacin meant specifically to exceed nutrient amounts and to act in a manner which was pharmacodynamically distinct from nutrient amounts of niacin. While Tang et al simply observes a correlation between micronutrient intake levels of niacin and then speculates that it may relate to a role for niacin in "immune function" [page 1252], the examples provided in the patent establish the unexpected finding that high doses of niacin given therapeutically to retrovirally infected patients with normal nutrient intakes results in improved tryptophan levels.

Knowledge of State of Art in June of 2000

21. Since 1991, I have been working as a physician an average of 60 hours per week. The predominant condition that I have treated since that time is retroviral infection.
22. Since 1991, I have been regularly reviewing publications related to infectious diseases. I typically have reviewed such publications on a regular basis, approximately once a week.
23. Since 1991, I have attended seminars and other informational meetings related to treating patients with retroviral infections on a regular basis, approximately eight to ten times a year.
24. I am familiar with the types of treatment that were available to patients infected with retroviruses in June of 2000.

Ability to practice the claimed invention using the '552 application as a guide.

25. I have recently reviewed the '552 application.
26. It is typical that patients with retroviral infections will periodically ask their physician to administer a "non-prescription" therapy, and physicians will then work with patients in an attempt to safely achieve the trial of therapy requested. In fact, in a recent study by Hsiao et al. over half of the retrovirally infected patients in the study were taking non-prescription therapies, and two-thirds of those patients discussed these therapies with their physicians. See Hsiao AF. et al. Complementary and Alternative Medicine Use and Substitution for Conventional Therapy by HIV infected Patients. J. Acquir Immune Defic Syndr. 2003 Jun 1; 33(2): 157-165.
27. Based upon my review, I could practice the invention using the '552 application as a guide for the following reasons:
 - a. The '552 application provides information on the administration of niacin and well as cites references for the reader to learn more information about the administration and effects of niacin. In addition, medical doctors or those in the medical field are often familiar with niacin. Those who are not readily familiar with niacin know that much information can be found regarding the administration and effects of niacin on "medline", in journals, books and other commonly available resources.
 - b. The '552 application tells me that the preferred method to combat plasma tryptophan depletion is to "administer niacin in 'pharmacological doses'". ('552 application, pg. 7, line 12).
 - c. The '552 application recommends that I administer a dose greater than 20 milligrams per day because a lesser dose would not be expected to produce the pharmacological

effect of combating plasma tryptophan depletion. ('552 application, pg 8, line 1).

- d. The '552 application informs me to expect pharmacological activity to occur at a dose of 100 milligrams per day. ('552 application, pg. 8, lines 10-11).
 - e. The '552 application informs me to expect that a patient will under go a reverse systemic tryptophan depletion upon the daily administration of 100 milligrams of niacin. ('552 application, pg. 8, lines 10-15).
 - f. The '552 application informs me that the preferred method of administration of niacin in this invention is oral administration. ('552 application, pg 9, lines 2-3).
 - g. The '552 application informs me that the preferred dose is 500 milligrams of niacin per day. ('552 application, pg. 9, lines 3-4).
 - h. The '552 application informs me that the preferred form of niacin to practice this invention is nicotinamide. ('552 application, pg. 9, lines 3-4).
 - i. By way of example, the '552 application informs me that administering 3 grams of nicotinamide per day for two months can be expected to increase plasma tryptophan between 20% and 80%. ('552 application, Table 3).
 - j. The known safe maximum dose for nicotinamide is 3 grams per day and this readily supported by the medical literature. *See, e.g., M. Knip et al., Safety of High-Dose Nicotinamide: A Review, 43 Diabetologia 1337-1345 (2000), a copy of which is attached as Exhibit E.*
 - k. The laboratory test to monitor plasma tryptophan concentrations is widely available.
 - l. The '552 application provides baseline systemic tryptophan levels that can be expected as well as expected increases in systemic tryptophan. ('552 application, Table 3). Furthermore, medical literature provides gives expected and target tryptophan levels. *See, e.g., Exhibit B.* Werner et al came up with 91 micromol/l as a baseline for systemic tryptophan from their study on tryptophan and HIV in 1988 *See Exhibit B.* Other studies have come up with different normal tryptophan levels - generally lower than 91 - but Werner and colleagues have stated their normal as 91 in three different studies. *See id.* Medical literature also establishes goal levels for tryptophan [by comparing tryptophan values in patients with HIV infection to healthy control patients] make this an easily administered pharmacological agent. *See, e.g., Exhibit B.*
28. As a doctor, I recognize that no two patients are the same. I also recognize that different patients react differently to the same treatment. Patients react differently to the same treatment for a myriad of reasons including different diets, different stress levels, different genetic makeup, and different metabolic rates. These observations make the determination

of optimal dosing of a drug in a particular case an individualized process.

29. If I were inclined to practice the invention disclosed in the '552 application, I would:

- a. In the ordinary case, confirm both retroviral infection and tryptophan depletion with simple blood tests, and then initiate treatment for tryptophan depletion by orally administering a daily dose of nicotinamide in the preferred amount of 500 milligrams per day.
- b. In a more extreme case of tryptophan depletion, I would initiate treatment by orally administering a daily dose of nicotinamide in the preferred amount of 3 grams per day.
- c. In either case, I would re-assess the patient periodically. If the tryptophan levels had increased I would maintain the treatment until tryptophan level had returned to an appropriate level. If the tryptophan level had not increased, I would raise the dosage commensurate with the condition.

30. In my opinion, no additional information or experimentation is needed to practice the invention.

'552 application is not directed to "antiviral" activity but is directed to alleviating systemic tryptophan depletion

31. The invention set forth in the '552 application is not directed to antiviral therapy. The invention set forth in the '552 application is directed to treating patients with systemic tryptophan depletion. In the study disclosed in the '552 patent (the '552 study), I found no evidence that the administration of niacin had any anti-viral effect.
32. While the '552 application does not include viral measurements of the patients involved in the '552 study, the '552 study did collect some information relating to patient viral load. Exhibit H is a summary that I prepared of the anti-viral data obtained in the '552 study. While the data set forth in Exhibit H is not conclusive scientific data, this early data from patient #1 lead to the impression that nicotinamide did not have a clear in vivo antiviral effect. Even though the one-week result looked somewhat favorable at the time, it turned out to be within the daily variations seen in this test in people not on antiviral therapy when all the data was viewed as a complete set. Given the emerging data I was developing at this time on increases in plasma tryptophan, the focus of the study became the tryptophan results and further viral load measurements were not obtained on this patient or the other three patients in the patent examples. On balance, the viral measurements I obtained were inconclusive. However, the lack of meaningful decrease in viral load on the only patient measured suggests, if anything, that the administration of niacin is not a direct anti-viral therapy at all. I am not aware of any *in vivo* study that has been able to demonstrate any anti-viral effect from niacin therapy. In any event, I am not claiming that the administration

of niacin has antiviral effects.

The 3 gms/day examples can be reasonably extrapolated to cover the claims

33. The '552 application provides statistically significant human clinical data with respect to the administration of 3 gms/day of niacin. *See* '552 application, Table 3. A normal dietary intake of niacin by one infected with a retrovirus can still leads to systemic tryptophan depletion in some cases. *See, e.g., Exhibit B.* Given this information, one knowledgeable in the field of infectious diseases could reasonably extrapolate the findings at 3 gms/day to a range in excess of a normal dietary intake of niacin.
34. The recommended daily dose of niacin is approximately 20 milligrams per day. *Id.* Thus, one would expect a favorable systemic tryptophan result between a range of 100 mgs/day and higher. *Id.*

The USPTO has already declared Murray et al (1995) as "non-enabled"

35. In 1995, I published two articles, both already made of record: (1) MF Murray, et al., Nicotinamide Inhibits HIV-1 in Both Acute and Chronic *In Vitro* Infection, Biochemical and Biophysical Research Communications, 210:954-959 (1995) and (2) MF Murray, et al., HIV Infection Decreases Intracellular Nicotinamide Adenine Dinucleotide [NAD], Biochemical and Biophysical Research Communications, 212:126-131 (1995). Collectively, these articles are referred to as the "1995 articles."
36. The 1995 articles were base on *in vitro* data. The same *in vitro* data that formed the basis of the 1995 articles also formed the basis of a patent application I filed with the United States Patent and Trademark Office ("USPTO"), U.S. patent application 07/906,689 (the "'689 application"). A true and correct copy of the '689 application is attached as Exhibit F.
37. The USPTO rejected the '689 patent application as unpatentable because it lacked *in vivo* substantiation, specifically:

The specifications provide data only for inhibiting HIV in cells in culture. There is no data to substantial the alleged utility for treating human subjects infected with HIV. There is no data to substantiate the alleged utility for treating human subjects infected with HIV.... Without statistically significant data documenting the claimed method for treating patients, the person of ordinary skill in the art, knowing the unpredictability of extrapolating from *in vitro* results to *in vivo* performance, would have good reason to doubt the efficacy of applicant's invention.

See Exhibit F, USPTO office action, page 2-3, rejections under §101 and § 112 (emphasis supplied). A true and correct copy of the August 25, 1992 office action is also attached as Exhibit F.

Brown Taught Treating Tryptophan Depletion with Tryptophan – Not Niacin

38. In his 1991 paper titled "Implications of Interferon-Induced Tryptophan Catabolism in Cancer, Auto-Immune Diseases and AIDS", Dr. RR Brown discussed the implications of tryptophan metabolism to HIV and AIDS. Dr. RR Brown recognized the importance of looking for a way to therapeutically intervene with respect to systemic tryptophan depletion. At no time in his 1991 paper – or anywhere else that I have found -- did Dr. Brown suggest tryptophan depletion could be treated with niacin.
39. In his 1991 paper, Dr. Brown hypothesized that decreased tryptophan might lead to decreased niacin (something that Skurnick later disproved). Dr. Brown also suggested treating tryptophan deficiency with tryptophan. Dr. Brown did not suggest niacin therapy for patients with HIV or other retroviral infections.
40. Dr. Brown's failure to suggest niacin cannot be considered an oversight since Dr. Brown is a prominent tryptophan researcher with a body of work encompassing over 100 articles stretching back to the 1950s [e.g. Brown RR, Vivian VM, Reynolds MS, et al. Some Aspects Of Tryptophan Metabolism In Human Subjects .2. Urinary Tryptophan Metabolites On A Low-Niacin Diet, J. Nutr 66 (4): 599-606 1958]
41. All statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true;
42. These statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. 1001, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Michael F. Murray
Michael F. Murray, M.D.

6/18/03
Date

**Treatment Of Retrovirus Induced Derangements With Niacin
Compounds**

5

FIELD OF INVENTION

This invention relates to the treatment of mammals chronically infected with
10 retroviruses, such as human immunodeficiency virus [HIV].

BACKGROUND

Retroviruses lead to chronic infection in mammals. Retroviruses are packets of
infectious nucleic acids (i.e. genetic material) surrounded by a protective protein coat.
15 Retroviruses are incapable of generating metabolic energy or synthesizing proteins, and
thus are characterized by dependence on living cells for replication and proliferation. A
retrovirus contains three enzymes: (1) reverse transcriptase, (2) protease, and (3)
integrase. Current antiviral drug therapy focuses on the inhibition of reverse transcriptase
and protease enzymes.
20 HIV is a prototypic retrovirus that causes the acquired immunodeficiency
syndrome [AIDS] in humans and related primates. Worldwide, AIDS has claimed over
11 million lives. HIV currently infects more than 30 million people. Since the first
reported cases of AIDS almost 20 years ago, the medical community has learned much
about this retroviral disease and its diverse manifestations. A number of clinical

manifestations of HIV infection, however, remain unexplained despite the efforts of the medical community to discover their etiology.

The Center for Disease Control and Prevention (the "CDC") has developed a "case definition" of the specific findings which, if present in a person with HIV, define AIDS. See Center for Disease Control and Prevention, *1993 Revised Classification System for HIV Infection and Expanded Surveillance Case Definition for AIDS Among Adolescents and Adults*, MMWR Morb Mortal Wkly Rep, 41(RR-17): 1-19(1992). The CDC's case definition falls into three broad categories: (1) CD4 immune cell depletion, (2) opportunistic infections, and (3) malignancies.

In addition to the case definition of AIDS, a number of metabolic changes are associated with this chronic infection. Among them are alterations in the circulating concentrations of amino acids. Amino acids are often referred to as the building blocks of proteins. Of the common amino acids, ten amino acids are "essential." The essential amino acids are those which the body cannot synthesize and therefore must be obtained directly through the diet.

Tryptophan, an essential amino acid, is known to be depleted during HIV infection. The body utilizes dietary-derived tryptophan for several important biochemical functions, including: (1) as a building block in the synthesis of proteins, (2) as a precursor of niacin and nicotinamide adenine dinucleotide [NAD], and (3) as a precursor of serotonin. Attempting to simply replete plasma tryptophan directly through pharmacologic doses of tryptophan is not advisable given the history of patients developing "eosinophilia myalgia syndrome."

Chronic retroviral infections lead to an ongoing metabolic burden on the infected subject. This burden in HIV infection includes: (1) the turnover of CD4 cells, (2) the disturbance of lipid metabolism, (3) the depletion of serotonin, (4) the depletion of plasma tryptophan [as discussed above], and (5) the depletion of intracellular NAD. The infection, over the course of months, leads to immunodeficiency (marked by CD4 depletion) and opportunistic infections. The infection also leads to a metabolic disease state marked by a number of other manifestations, including a non-specific “wasting syndrome” and the specific disturbances and depletions previously mentioned in this paragraph.

Presently, no cure exists for HIV infection. Current treatments for HIV infected patients tend to focus on agents which inhibit two viral enzymes: the HIV-reverse transcriptase [reverse transcriptase inhibitors] or the HIV-protease [protease inhibitors]. Such agents include among others, ZDV (zidovudine), DDI (2'-3' -dideoxyinosine), and DDC (2' -3' -dideoxycytidine), each of which blocks the HIV proliferation in cells (ZDV, DDI , DDC and other such agents are referred to as the “licensed antivirals”).

Unfortunately, the inhibition which occurs with the licensed antivirals is incomplete. Over time, HIV becomes resistant to the licensed antivirals. This resistance can result in a resumption of progressive immune system destruction.

Zidovudine, a licensed antiviral compound, is the only compound known to replete plasma tryptophan in HIV infected persons. However, zidovudine which is a reverse transcriptase inhibitor, causes a number of side effects including headache, nausea, and bone marrow suppression. Furthermore, HIV can develop resistance to

Zidovudine, an event which would be expected to result in recurrent tryptophan depletion.

Since HIV depletes plasma tryptophan and since this essential amino acid is required in a range of biologically necessary tasks, replenishing plasma tryptophan is essential in maintaining overall health in the HIV infected state. Although the antiviral drug zidovudine leads to an increase in plasma tryptophan in HIV infected persons, this reversal would be expected to last only so long as virus inhibition persists, and antiviral drug failure is expected with time given the incomplete nature of the drug's inhibitory effect. Niacin, as an agent to reverse infection-induced metabolic changes, works on the host side of the virus-host interaction and therefore would not be subject to the same risk of eventual viral drug resistance.

BRIEF SUMMARY OF THE INVENTION

This invention inhibits adverse metabolic and immunologic effects associated with chronic retroviral infections such as HIV by using niacin compounds, such as nicotinamide or nicotinic acid, to inhibit the depletion of tryptophan and to induce the restoration of intracellular nicotinamide nucleotides, such as nicotinamide adenine dinucleotide [NAD], in patients with retroviral infections.

More particularly, this invention relates to the oral use of pharmacologic doses of niacin compounds in persons with HIV infection in order to reverse or prevent deleterious metabolic consequences of the infection.

Another object of the invention is to inhibit adverse effects of HIV infection by combining the method of this invention with known HIV inhibitors, such as reverse transcriptase inhibitors, protease inhibitors, and others.

The invention provides a method of administering a therapeutically effective
5 amount of niacin compounds to a patient with a chronic retroviral infection such as HIV, the etiological agent clinically associated with AIDS.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

Table 1 - Baseline Characteristics of Niacin Study Patients. Illustrates the
10 immunological status as measured by CD4 count, the concomitant use of antiviral medications, and the presence of co-infections. Niacin worked to improve tryptophan status in all four patients across this range of baseline infectious disease related findings.

Table 2 - Baseline Dietary intake of Niacin Study Patients. Illustrates the range of
baseline dietary intake of tryptophan and niacin compounds. The amounts were
15 determined by dietary recall survey, and demonstrate that tryptophan and niacin were not deficient in the baseline diet of these patients, and that the pharmacological dose of niacin used in the study was significantly higher than all participant's baseline intake.

Table 3 - Changes in plasma tryptophan levels [micromols/l] in patients taking 3
gram of nicotinamide daily for 2 months. The increase in the levels of this essential
20 amino acid despite the unchanged dietary intake of tryptophan is consistent with decreased metabolic shunting of essential tryptophan towards niacin in HIV infected persons.

Table 4 - Changes in non-tryptophan plasma amino acid levels in HIV patients taking 3 grams/day of oral nicotinamide. The four amino acids include two essential amino acids [methionine and lysine] and two nonessential amino acids [cysteine and taurine]. In all four cases there is no discernible pattern of change with this intervention, supporting the observation that the effect of pharmacological doses of niacin on plasma tryptophan is a specific and important intervention against the metabolic disruption caused by HIV infection.

DESCRIPTION

The invention is a method for treatment of HIV infected persons with niacin administered in an amount effective to combat plasma tryptophan depletion. This invention is useful for any mammal infected with a retrovirus, including HIV. Through administration of a pharmacological dose of niacin, the retrovirus-infected subject's systemic tryptophan depletion will be reversed.

Niacin refers to either of two chemically related compounds: nicotinamide or nicotinic acid. Niacin may be administered orally, parenterally, rectally, or with any pharmaceutically accepted adjuvant or carrier. The administration and effects of niacin have undergone extensive study in the fields of diabetes and hypercholesterolemia. (See, e.g., Petley A, *et al*, *The Pharmacokinetics of Nicotinamide in Humans and Rodents*, *Diabetes*, 44: 152-155 (1995); and DiPalma JR and Thayer WS, *Use of Niacin as a Drug*, *Annu. Rev. Nutr.*, 11:169-87, (1991)). Niacin, or vitamin B3, is the common name for both nicotinic acid, i.e., C₆H₅N₀O₂, (pyridine-3-carboxylic acid) or nicotinamide, i.e., C₆H₆N₂O₂ (3-pyridinecarboxamide).

Niacin is a precursor to the biosynthesis of nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP). Nicotinamide nucleotides (NAD and NADP) participate in a wide array of oxidation-reduction reactions catalyzed by dehydrogenase or oxido-reductase enzymes. Virtually every aspect of cellular metabolism involves NAD/NADH or NADP/NADPH dependent reactions. In absence of sufficient supplies of nicotinamide nucleotides or niacin precursors for nicotinamide nucleotide biosynthesis, cellular functions and life itself would be impaired. (DiPalma JR and Thayer WS, *Use of Niacin as a Drug*, Annu. Rev. Nutr., 11:169-87, (1991)). The body can readily convert nicotinic acid to nicotinamide and both are expected to produce the desired therapeutic effect of combating plasma tryptophan depletion.

For this invention, it is preferred to administer niacin in "pharmacologic doses." A vitamin compound is considered a "drug," not a "nutrient," when: [1] the ingested dose exceeds the dose required for nutrient function, and [2] a pharmacologic action distinct from nutrient function is achieved. Maintaining plasma tryptophan is not a nutrient function of niacin; rather, it is a pharmacological action of niacin in retrovirally infected subjects.

All vitamins fill a nutrient function whereby a sufficient amount of the vitamin compound is required in the diet to fulfill normal metabolic needs. The body normally requires 12-18 milligrams of niacin per day to carry out the coenzyme function which defines niacin as a vitamin. The Recommended Daily Allowance [RDA] of niacin is approximately 13-20 milligrams per day. Therefore, a non-pharmacologic dose of niacin,

where niacin acts as a vitamin or nutrient compound, is approximately 20 milligrams a day or less.

The use of pharmacologic doses of niacin is distinct from the vitamin or nutrient use of niacin. (DiPalma JR and Thayer WS, *Use of Niacin as a Drug*, Annu. Rev. Nutr., 5 11:169-87, (1991)). Niacin's pharmacologic use can be distinguished from its non-pharmacologic (or physiologic) use by the pharmacodynamic action of the compound. Pharmacodynamic action begins when the nutrient function of niacin is complete. The maintenance of plasma tryptophan in the face of (1) retrovirus infection, and (2) normal or supernormal niacin levels is the distinct pharmacodynamic action described here.

10 A pharmacological dose of niacin generally occurs at a dose of about 100 milligrams per day, about 5 times the recommended daily allowance [RDA]. Niacin is safe in doses greater than 100 mg in persons with HIV, and doses of greater than 100 mg should also cause a retrovirus-infected patient to undergo a reverse systemic tryptophan depletion.

15 Because pharmacologic doses of niacin alleviate the drive to deplete plasma tryptophan, tryptophan depletion may represent a metabolic shunt towards niacin production. (See Murray, *Niacin as a Potential AIDS Preventative Factor*, Medical Hypotheses 53(5), 375-379 (November 1999), which is incorporated herein by reference.) In addition, because the essential amino acid tryptophan cannot be synthesized in the 20 body, any agent which increases in the circulating concentrations of tryptophan in HIV infected persons presumably does so by diminishing the metabolic demands on the available supply.

The preferred embodiment of this invention is to administer a mammal infected with a retrovirus with niacin. The preferred method of administration is oral administration. The preferred dose is 500 milligrams of niacin per day in the form of nicotinamide.

5 The following EXAMPLE is presented to more fully illustrate the preferred embodiment of the invention. The example should not be construed to limit the scope of the invention and is to be understood merely for the purpose of illustration.

EXAMPLE - Clinical Trial of Niacin in HIV infected persons.

10 Four HIV infected persons participated in a trial of niacin in the form of nicotinamide. The participants were at various stages of their HIV infection as judged by their CD4 counts which ranged from 0 to 620 [see table 1]. The participants were receiving either a stable regimen of anti-viral drugs [i.e. anti-HIV drugs] for a period greater than one year or were not taking any anti-viral drugs. Two of the participants had known co-infections typical of HIV
15 infected persons. Each participant took 3 grams of nicotinamide per day for 2 months. This treatment was not associated with any adverse side effects. Each participant's plasma tryptophan was measured prior to treatment and at the end of treatment [see table 3]. The average increase of plasma tryptophan of all participants was 43.9%. This change in tryptophan concentration was statistically
20 significant with a calculated p value of $p=0.0112$ [using paired t-test]. The study also measured 4 other plasma amino acids which are listed in table 4. All amino acid concentrations were measured by High Performance Liquid Chromotography [HPLC]. There was no significant change in the plasma amino

acid concentrations other than tryptophan. As demonstrated in tables 3 and 4, only plasma tryptophan changed in a statistically significant manner.

The details of the invention have been set forth in the accompanying description
5 and example above. Although any methods and materials similar or equivalent to those
described herein can be used in the practice or testing of the present invention, the
preferred methods and materials have been described. Other features, object, and
advantages of the invention will be apparent from the description and from the claims. In
the specification and the claims, the singular forms include plural referents unless the
10 context clearly requires otherwise. Unless defined otherwise, all technical and scientific
terms used herein have the same meaning as commonly understood by one of ordinary
skill in the art to which this invention belongs. All patents and publications cited in this
specification are incorporated by reference.

CLAIMS

What is claimed is:

1. A method for treating a patient infected with a retrovirus, which comprises the step of administering a daily pharmacological dose of niacin.
- 5 2. A method for treating retrovirus-induced metabolic changes, which comprises the step of administering a daily pharmacological dose of niacin.
3. A method for treating a patient infected with HIV, which comprises the step of administering a daily pharmacological dose of niacin.
- 10 4. A method for treating HIV-induced metabolic changes, which comprises the step of administering a daily pharmacological dose of niacin.
5. A method for treating retrovirus-induced metabolic changes in a patient's systemic tryptophan levels, which comprises the step of administering a daily pharmacological dose of niacin.
- 15 6. A method for treating HIV-induced metabolic changes in a patient's systemic tryptophan levels, which comprises the step of administering a daily pharmacological dose of niacin.
7. A method for treating the depletion of tryptophan in a retrovirus-infected patient, which comprises the step of administering a daily pharmacological dose of niacin.
- 20 8. A method for treating the depletion of tryptophan in an HIV-infected patient, which comprises the step of administering a daily pharmacological dose of niacin.

9. A method for replenishing nicotinamide nucleotide precursors, which comprises the step of administering a daily pharmacological dose of niacin.
10. The method of claim 1, 2, 3, 4, 5, 6, 7, 8, or 9, where said dose is of an amount sufficient to prevent retrovirus-induced metabolic changes in systemic tryptophan concentrations.
11. The method of claim 1, 2, 3, 4, 5, 6, 7, 8, or 9, where said dose is of an amount sufficient to slow down the rate of retrovirus-induced metabolic changes in systemic tryptophan concentrations.
12. The method of claim 1, 2, 3, 4, 5, 6, 7, 8, or 9, where said dose is of an amount sufficient to stop the rate of retrovirus-induced metabolic changes in systemic tryptophan concentrations.
13. The method of claim 1, 2, 3, 4, 5, 6, 7, 8, or 9, where said dose is of an amount sufficient to increase a patient's level of plasma tryptophan.
14. A method as in claim 1, 2, 3, 4, 5, 6, 7, 8, or 9, where said dose is greater than 100 milligrams per day.
15. A method as in claim 1, 2, 3, 4, 5, 6, 7, 8, or 9, where said dose is approximately 3 grams per day.
16. A method as in claim 1, 2, 3, 4, 5, 6, 7, 8, or 9, where said dose exceeds the standard recommended daily amounts for coenzyme activity.
17. A method of claim 1, 2, 3, 4, 5, 6, 7, 8, or 9, where said dose exceeds amounts normally obtainable with routine diet and supplement practices.

18. A method as in claim 1, 2, 3, 4, 5, 6, 7, 8, or 9, where said dose exceeds the RDA [recommended daily allowance] of niacin.
19. A method as in claim 1, 2, 3, 4, 5, 6, 7, 8, or 9, where said dose is sufficient to raise the intracellular levels of nicotinamide adenine dinucleotide [NAD] in persons with HIV infection.
20. A method as in claim 1, 2, 3, 4, 5, 6, 7, 8, or 9, where said dose is sufficient to replete nicotinamide nucleotide precursors [NAD].
21. A method of claim 1, 2, 3, 4, 5, 6, 7, 8, or 9, where said dose of niacin is administered to persons with HIV and other co-infections.
22. A method as in claim 1, 2, 3, 4, 5, 6, 7, 8, or 9, where said dose of niacin is administered in combination with antiviral medications such as reverse transcriptase inhibitors, and protease inhibitors.
23. A method as in claim 1, 2, 3, 4, 5, 6, 7, 8, or 9, where said dose is administered in combination with other treatments for HIV infection to improve the metabolic status of an infected patient.
24. A method as in claim 1, 2, 3, 4, 5, 6, 7, 8, or 9, where said dose is sufficient to inhibit new virus production.

ABSTRACT OF THE DISCLOSURE

Chronic infection with retroviruses, such as HIV, induce a number of metabolic derangements. The present invention relates to a method for treating retrovirus-infected subjects with niacin compounds to reverse infection induced metabolic derangements.

5

DRAWINGS

Table 1 - Baseline Infectious Disease Characteristics of Nicotinamide Study Patients.

5

Patient	CD4 count	Antiretroviral [duration]	Co-infections
1	0	none	mollusum contagiosum
2	220	PI ¹ /RTI ² [3 years]	none
3	290	RTI [2 years]	none
4	620	none	herpes zoster

**Table 2 - Baseline Dietary Characteristics of Nicotinamide Study Patients. Daily intake
for tryptophan and niacin by dietary survey**

10 [i.e. these numbers reflect the total non-pharmacologic amounts included in participants
food and nutritional supplements.]

Patient	Tryptophan [daily intake]	Niacin [RDA %]
1	0.89 gms	42.0 mg [210%]
2	1.44 gms	22.4 mg [112%]
3	0.66 gms	32.8 mg [164%]
4	1.05 gms	24.0 mg [120%]

15

¹ PI is protease inhibitor.

² RTI is reverse transcriptase inhibitor.

Table 3 - Changes in plasma tryptophan levels [micromols/l] in patients taking 3 gram of nicotinamide daily for 2 months.

Patient	Days of Treatment	Baseline Plasma Tryptophan	Final Plasma Tryptophan	Change in Plasma Tryptophan
1.	57	31.1	52.9	+ 70.1%
2.	61	53.4	82.3	+ 54.1 %
3.	63	62.0	75.1	+ 21.1%
4.	60	51.0	66.5	+ 30.4%

Table 4 - Changes in non-tryptophan plasma amino acid levels in HIV infected patients taking 3 grams/day of oral nicotinamide.

Patient	Days of Treatment	Baseline Plasma Methionine	Final Plasma Methionine	Change in Plasma Methionine
1.	57	19.8	18.3	- 7.6%
2.	61	15.6	17.1	+ 9.6 %
3.	63	34.3	24.4	- 28.9%
4.	60	18.3	20.4	+ 11.5%

5

Patient	Days of Treatment	Baseline Plasma Lysine	Final Plasma Lysine	Change in Plasma Lysine
1.	57	218.7	111.1	- 49.2%
2.	61	97.7	141.2	+ 44.5 %
3.	63	251.8	162.7	- 34.5%
4.	60	191.8	129.1	- 32.7%

Patient	Days of Treatment	Baseline Plasma Cysteine	Final Plasma Cysteine	Change in Plasma Cysteine
1.	57	48.3	54.7	+ 13.3%
2.	61	27.0	28.8	+ 6.6 %
3.	63	35.6	39.1	+ 9.8%
4.	60	75.5	61.3	-18.8%

Table 4 (cont.)

Patient	Days of Treatment	Baseline Plasma Taurine	Final Plasma Taurine	Change in Plasma Taurine
1.	57	46.3	68.8	+ 48.6%
2.	61	76.2	87.4	+ 14.4 %
3.	63	92.1	69.7	- 24.3%
4.	60	80.6	61.6	- 23.6%

Table 1 – Studies Examining Plasma or Serum Tryptophan in HIV infected patients

Tryptophan concentration	Intervention	Other Measures	Comments	Reference [year]
44.8 $\mu\text{mol/l}$ in infected patients verses 91.0 $\mu\text{mol/l}$ in controls	None specified	KT ratio was 3:1 in patients verses controls	Increased KT ratio suggests that increased TO not dietary or other types of loss explain lower concentration	Werner et al. [1988]
28.4 $\mu\text{mol/l}$ in infected patients verses 39.7 $\mu\text{mol/l}$ in controls	No patients on antiretroviral medications	CSF tryptophan 1.52 $\mu\text{mol/l}$ in infected patients verses 2.18 $\mu\text{mol/l}$ in controls	Lower tryptophan concentrations most pronounced at low CD4 counts	Larsson et al. [1989]
48.8 $\mu\text{mol/l}$ in infected patients with dementia or neuropathy, 70.5 $\mu\text{mol/l}$ in patients without dementia or neuropathy, and 91.1 $\mu\text{mol/l}$ in controls	None specified	Neopterin concentrations have a reciprocal relationship to tryptophan concentrations	Neurological findings correlated with lower tryptophan concentrations. (Note - same control group as Werner et al.)	Fuchs et al. [1990a]
29.8 $\mu\text{mol/l}$ in infected patients verses 39.7 $\mu\text{mol/l}$ in controls	None specified	Serum IFN γ levels were 159 U/liter in patient serum verses 33 U/liter in control serum.	Inverse correlation between tryptophan and IFN γ concentrations noted.	Fuchs et al. [1990b]
57 $\mu\text{mol/l}$ in infected patients verses 91 $\mu\text{mol/l}$ in controls	38% of patients on ZDV monotherapy	IFN γ 259 U/l in infected patients and 23.5 U/l in controls. Kynurine 3.45 $\mu\text{mol/l}$ in infected patients verses 2.31 $\mu\text{mol/l}$ in controls.	P<0.001 for the inverse correlation between tryptophan and IFN γ concentrations. No separate analysis based on antiviral therapy.	Fuchs et al. [1991]
40.2 $\mu\text{mol/l}$ in infected patients verses 70.9 $\mu\text{mol/l}$ in controls	No patients on antivirals	Tryptophan decreases accompanied proportional increases in kynurenine and quinolinic acid in both serum and CSF	Elevated concentrations of the neurotoxic intermediate QA demonstrated.	Heyes et al. [1992]
22 $\mu\text{mol/l}$ in infected patients verses 46 $\mu\text{mol/l}$ in controls	85% of patients on mono or dual nucleoside therapy	Decrease cystine, trypto, methionine, increased taurine, lysine	Trypto and lysine showed trend of lower/higher with cd4 less than 200. No separate analysis based on	Hortin et al. [1994]

51 $\mu\text{mol/l}$ in infected patients versus 59 $\mu\text{mol/l}$ in controls	None specified	Plasma concentrations of amino acids pre and post IV infusion of amino acids.	antiviral therapy Despite lower concentrations, tryptophan does not appear to be rate limiting in protein synthesis in AIDS patients.	Laurichesse et al. [1998]
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MURRAY
1988

Tryptophan Degradation in Patients Infected by Human Immunodeficiency Virus

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(Received 15 February 1988)

Summary: Tryptophan and kynurenine were measured retrospectively in sera of 11 male patients with advanced human immunodeficiency virus (HIV) infection (Walter Reed stages 4 and 6). Tryptophan levels are significantly reduced to less than 50% in patients with HIV infection and kynurenine levels significantly elevated when compared to sex and age matched

controls. The decrease of tryptophan levels might contribute to neurologic symptoms often associated with HIV infection. Since interferon- γ induces degradation of tryptophan via the kynurenine pathway, the present results may be consistent with enhanced endogenous production of interferon- γ in advanced HIV infection.

Tryptophan-Abbau bei HIV-infizierten Patienten

Zusammenfassung: Tryptophan und Kynurenin wurden retrospektiv im Serum von 11 Patienten (alle männlich) mit fortgeschrittener HIV-Infektion (Walter-Reed-Stadium 4 bzw. 6) bestimmt. Tryptophan war verglichen mit Gesunden gleichen Geschlechts und Alters signifikant erniedrigt und Kynurenin erhöht. Die beobachteten niedrigen Tryptophanwerte könnten zu den

neurologischen Symptomen beitragen, die häufig bei HIV-Infizierten auftreten. Da Interferon- γ den Tryptophanabbau über Kynurenin als Zwischenprodukt induziert, sind unsere Ergebnisse mit Berichten über vermehrte Produktion von Interferon- γ bei Patienten mit fortgeschrittener HIV-Infektion vereinbar.

Key words: Degradation of tryptophan, kynurenine, neopterin, infection by human immunodeficiency virus, endogenous interferon- γ .

A great deal of evidence has accumulated within the last few years demonstrating that early steps of T-cell macrophage interaction are activated in patients with AIDS. This observation is supported by several findings among them highly elevated levels of neopterin, a product of interferon- γ -activated macrophages, in urine^[1], serum^[2,3] and cerebrospinal fluid^[4] of patients

with AIDS. In addition, interferon- γ was detected in sera of AIDS patients^[5]. Interferon- γ is known to induce tryptophan degradation via the kynurenine pathway. In vitro^[6] as well as in vivo^[7,8] the degradation of tryptophan correlates to increased synthesis of neopterin. Thus, degradation of tryptophan via the kynurenine pathway can be expected to occur in patients

Abbreviations:

HIV, human immunodeficiency virus; AIDS, acquired immunodeficiency syndrome; ARC, AIDS-related complex; WR, Walter Reed Staging classification; HPLC, high-performance liquid chromatography.

with advanced HIV infection. We have examined retrospectively tryptophan and kynurenine concentrations in sera of patients infected by HIV and compared to neopterin concentrations. We have found significant decrease of tryptophan levels in patients which, by its impact on brain serotonin metabolism, might contribute to neurologic symptoms often associated with HIV infection.

Patients and Methods

Eleven male patients, aged 39 ± 10 years, were classified to stages WR4 ($n = 5$) and WR6 ($n = 6$) according to the Walter Reed staging classification^[9]. The control group consisted of eleven male blood donors, aged 40 ± 12 years. Kynurenine, neopterin and creatinine were analysed in sera by HPLC^[10]. Kynurenine was detected by fluorescence (excitation 353 nm, total emission) and ultraviolet absorption (260 nm) with a detection limit of $1 \mu\text{mol/l}$, neopterin and creatinine were determined as described^[10]. The sum of free and protein-associated, but not covalently bound tryptophan, was measured in serum diluted with a ten-fold volume of aqueous 0.9% sodium chloride by HPLC as described^[6]. *P*-values for statistical significance were computed by Student's *t*-test and confirmed by Mann Whitney *U*-test. All figures shown are means \pm standard deviations.

Results

Comparing patients to controls, serum levels of tryptophan are decreased to less than 50% and serum levels of kynurenine are elevated. Thus, the ratio of tryptophan per kynurenine drops by a factor of three (see table). Mean levels of neopterin in serum are eight times higher in patients than in controls. All differences are highly significant (see table). The degree of significance remains unchanged when the levels are

related to the concomitantly measured creatinine, which is lower in the patients with HIV-related disease ($70.9 \pm 11.7 \mu\text{mol/l}$) than in the controls ($101.7 \pm 17.6 \mu\text{mol/l}$). The figure displays the individual concentrations of tryptophan, kynurenine and neopterin. Whereas kynurenine levels show some overlap, all tryptophan levels are lower and all neopterin levels are higher in patients compared to controls.

Discussion

The increase of kynurenine and the decrease of tryptophan, which remained significant upon relating levels to creatinine, provide evidence that the lower tryptophan levels of the patients infected by HIV originate from tryptophan degradation via the kynurenine pathway rather than from secondary phenomena such as altered utilization of dietary tryptophan. The finding of increased levels of arginine and glutamate^[11] underlines that the depletion of tryptophan reflects a specific phenomenon rather than a general decrease of amino-acid levels in HIV-infected patients. Since interferon- γ induces tryptophan degradation via the kynurenine pathway^[6-8], the data presented in this work may be consistent with enhanced endogenous interferon- γ production in advanced infection by HIV^[5]. The extent of the degradation of tryptophan and of raised levels of neopterin found in HIV-infected patients correspond to results obtained after administration of interferon- γ to cancer patients^[8].

The observed catabolism of tryptophan might contribute to neurologic symptoms often accompanying HIV infection by withdrawal of the tryptophan required for serotonin synthesis.

Table. Tryptophan, kynurenine and neopterin concentrations in sera of AIDS and ARC patients compared to blood donors (figures are means \pm standard deviation).

	Patients	Controls	<i>P</i> *
Numbers of subjects	11	11	
Age	39 ± 10	40 ± 12	
Tryptophan [$\mu\text{mol/l}$]	44.8 ± 8.4	91.0 ± 22.0	< 0.0001
Kynurenine [$\mu\text{mol/l}$]	3.53 ± 0.89	2.31 ± 0.77	0.003
Tryptophan/kynurenine	13.4 ± 3.7	42.5 ± 13.7	< 0.0001
Neopterin [nmol/l]	39.1 ± 17.0	4.5 ± 1.5	< 0.0001

* Student's *t*-test.

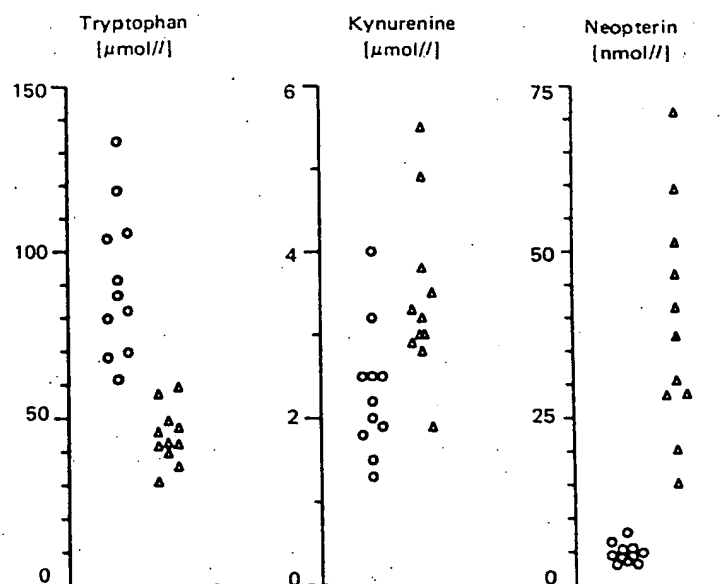


Figure. Serum concentrations of tryptophan, kynurenine and neopterin.

○ Blood donors; △ patients infected by HIV.

The concentrations of serotonin in brain physiologically depend on plasma tryptophan levels^[12], which we show here to be substantially decreased in advanced infections by HIV. Further, several findings render plausible that in addition to the observed decrease of tryptophan levels in peripheral blood, tryptophan will also be degraded in the central nervous system. Immunologic reactions against HIV in the brain have been demonstrated in AIDS patients with neurologic symptoms^[13]. It is to be anticipated that this reactions might be accompanied by increased endogeneous production of interferon- γ in the brain. This assumption is supported by extremely high neopterin levels observed in the cerebrospinal fluid of AIDS patients^[4]. Thus, in addition to the impaired supply of tryptophan from the periphery, further decrease of tryptophan likely occurs in the brain in case of intracerebral viral infection leading to a severely impaired brain serotonin metabolism, which is associated with a variety of neurologic symptoms^[14-16]. We suggest, therefore, that the disorder of the brain serotonin metabolism due to decrease of tryptophan levels should be considered as a potential source of neurologic symptoms in patients infected by HIV. The proposed mechanism can easily explain several features of HIV-related neurologic symptoms, for example the striking coincidence of acute encephalopathy with seroconversion for HIV^[17]. To what extent concomitant infections contribute in addition to HIV to the tryptophan degradation observed here remains to be elucidated. In any event, our observations might provide a rationale for the therapy of neurologic symptoms in AIDS.

This work was supported by the *Austrian Research Funds „Zur Förderung der wissenschaftlichen Forschung“*, project P6036M.

Literature

- 1 Wachter, H., Fuchs, D., Hausen, A., Huber, C., Knosp, O., Reibnegger, G. & Spira, T.J. (1983) *Hoppe-Seyler's Z. Physiol. Chem.* 364, 1345-1346.
- 2 Kern, P., Rokos, H. & Dietrich, M. (1984) *Biomed. Pharmacother.* 38, 407.
- 3 Fuchs, D., Reibnegger, G., Wachter, H., Jäger, H., Popescu, M. & Kaboth, W. (1987) *Ann. Intern. Med.* 107, 784-785.
- 4 Smith, I., Howells, D.W., Kendall, B., Levinsky, R. & Hyland, K. (1987) *Lancet* ii, 215.
- 5 Harrer, T., Messing, K., Bienzle, U., Meyer, E., Giedl, J. & Kalden, J.R. (1987) *Klin. Wochenschr.* 65, 864-872.
- 6 Werner, E.R., Bitterlich, G., Fuchs, D., Hausen, A., Reibnegger, G., Szabo, G., Dierich, M.P. & Wachter, H. (1987) *Life Sci.* 41, 273-280.
- 7 Byrne, G.I., Lehmann, L.K., Kirschbaum, J.G., Borden, E.C., Lee, C.M. & Brown, R.R. (1986) *J. INF. Res.* 6, 389-396.
- 8 Datta, S.P., Brown, R.R., Borden, E.C., Sondel, P.M. & Trump, D.L. (1987) *Proc. Am. Assoc. Cancer Res.* 28, 338.
- 9 Redfield, R.R., Wright, D.C. & Tramont, E.C. (1986) *N. Engl. J. Med.* 314, 131-132.
- 10 Werner, E.R., Fuchs, D., Hausen, A., Reibnegger, G. & Wachter, H. (1987) *Clin. Chem.* 33, 2028-2033.
- 11 Dröge, W., Eck, H.P., Näher, H., Pekar, U. & Daniel, V. (1988) *Biol. Chem. Hoppe-Seyler* 369, 143-148.
- 12 Fernstrom, J.D. & Wurtman, R.J. (1971) *Science* 173, 149-152.
- 13 Resnick, L., DiMarzo-Veronesse, F., Schüpbach, J., Tourtellotte, W.W., Ho, D.D., Müller, F., Shapshak, P., Vogt, M., Groopman, J.E., Markham, P.D. & Gallo, R.C. (1985) *N. Engl. J. Med.* 313, 1498-1504.

- 14 Stanley, M. & Mann, J.J. (1983) *Lancet* i, 214-216. 17 Carne, C.A., Smith, A., Elkington, S.G., Preston, F.E., Tedder, R.S., Sutherland, S., Daly, H.M. & Craske, J. (1985) *Lancet* ii, 1206-1208.
15 H.M. von Praag (1982) *Lancet* ii, 1259-1264.
16 Anonymus (1987) *Lancet* ii, 949-950.

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Indole Amine Deficiency in Blood and Cerebrospinal Fluid From Patients With Human Immunodeficiency Virus Infection

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Twenty-four patients with human immunodeficiency virus (HIV) infection were investigated for possible changes in certain indole amine constituents in blood and cerebrospinal fluid (CSF). Albumin in serum was determined and used as a rough nutritional marker. Six of the 24 patients had acquired immunodeficiency syndrome AIDS, four had other clinical symptoms of HIV infection, and 14 had no apparent symptoms. The HIV-seropositive patients had significantly decreased tryptophan values; their blood concentrations were 28% lower and their CSF concentrations 30% lower than corresponding values in 14 healthy controls. The blood concentrations of 5-hydroxytryptamine (5-HT) were 50% lower, and the platelet content of 5-HT was 36% lower in HIV-infected individuals than in the control group. The most pronounced changes were invariably seen in the six cases with AIDS and in patients with a low number of CD4⁺ cells. No significant difference between controls and HIV-seropositive patients was detected in the mean CSF concentrations of 5-hydroxyindoleacetic acid (5-HIAA), although these levels were markedly reduced in four of the HIV patients. Neither was any significant difference seen between patients and controls in the serum concentrations of albumin.

Key words: HIV, AIDS, tryptophan, 5-HT, 5-HIAA, blood, CSF

INTRODUCTION

Symptoms of the central nervous system (CNS), diarrhea, and acquired immune deficiency syndrome (AIDS) cachexia ("slim disease") are common in the advanced stages of acquired immunodeficiency syndrome but may also appear as primary manifestations of HIV infection in man (Navia et al., 1986; Serwadda et al., 1985). These symptoms are often seen in the absence of any concurrent illness other than human immunodeficiency virus (HIV) infection to explain the findings.

CD4⁺ helper/inducer T-lymphocytes are the pri-

mary target for HIV, but the virus has also been detected in brain tissue macrophages (Koenig et al., 1986), in cerebrospinal fluid (CSF), (Ho et al., 1985; Åsjö et al., 1986; Chiodi et al., 1988), and in gastrointestinal epithelium (Nelson et al., 1988). Because, however, only a very small fraction of all CD4⁺ cells appear to be productively infected with HIV, it is unlikely that direct viral destruction is a pathogenic mechanism for all symptoms associated with the disease (Ho et al., 1987). Thus, the evidence suggests that secondary effects of the infection cause some of the symptoms.

Assuming that HIV-induced changes in the brain and in the gastrointestinal tract might be reflected by abnormal concentrations of blood and CSF constituents associated with diseases in these tissues, we have analyzed some indole amine compounds in HIV patients and healthy controls. The blood concentrations of tryptophan and 5-hydroxytryptamine (5-HT) and the CSF concentrations of tryptophan and 5-hydroxyindoleacetic acid (5-HIAA) were determined. The number of platelets was studied in relation to the blood levels of 5-HT, and the number of helper cells was assessed in blood. Serum concentrations of albumin were determined for use as a rough marker reflecting the nutritional status of the patients.

MATERIAL AND METHODS

Subjects

The study involved 24 HIV-seropositive patients, 21 men and three women, aged 19-69 years (mean age 35 years), living in the city of Göteborg (Sweden). Six had AIDS, as defined in accordance with the surveillance criteria of the Center for Disease Control (CDC). Other clinical symptoms of HIV infection were diagnosed in

Received November 10, 1988; revised March 22, 1989; accepted March 24, 1989.

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EXHIBIT B

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TABLE I. Clinical Data in 24 Patients With HIV Infection

	Sex/age	Years with HIV	Risk factor	Somatic complications	Psychiatric complications	Number of helper cells ^a
Patients with AIDS						
Case 1	M/31	?	Homosexuality	Pneumocystis	Dementia	<0.02
Case 2	M/40	?	Homosexuality	Pneumocystis, Kaposi's sarcoma		0.02
Case 3	M/37	?	Homosexuality	Pneumocystis	Dementia	0.02
Case 4	M/26	?	Homosexuality	Pneumocystis		0.02
Case 5	M/45	>4	Homosexuality	Pneumocystis		
Case 6	M/58	1.5	Homosexuality	Lymphoma		0.29
Symptomatic patients						
Case 7	M/69	1	Transfusion	GB, myeloplastic disorder		0.06
Case 8	M/49	0.5-1	Homosexuality	LAS		
Case 9	M/26	?	Homosexuality	LAS		0.19
Case 10	M/33	2	Homosexuality	ARC (diarrhea)		0.53
Asymptomatic patients						
Case 11	M/21	>4	Homosexuality		Dementia, psychosis	0.32
Case 12	M/28	0.5	Homosexuality			0.56
Case 13	F/37	1.5	Transfusion			0.38
Case 14 ^b	M/25	3				0.46
Case 15	M/25	2-3	Homosexuality			0.71
Case 16	M/24	?	Homosexuality	Condyloma		1.29
Case 17	M/33	?	Homosexuality	Nonpurulent coxarthrit		0.37
Case 18	M/31	?	Homosexuality			2.10
Case 19	M/40	?	Homosexuality			0.46
Case 20 ^b	F/28	5				0.12
Case 21	M/47	1	Homosexuality			0.56
Case 22	M/39	>2	Homosexuality		Dyslexia, dysgraphia	0.34
Case 23	F/19	?	Drug addiction			0.38
Case 24	M/25	0.5	Homosexuality	Aseptic meningitis		0.90

^aTotal number of helper cells ($\times 10^9$ /liter).^bHeterosexual transmission.

four patients: two with lymphadenopathy syndrome (LAS), one with Guillain Barré's syndrome (GB), and one with AIDS-related complex (ARC) (*symptomatic* cases). Fourteen patients were seropositive for HIV antibodies but showed no somatic symptoms of HIV infection (*asymptomatic* cases). Dementia or other neuropsychiatric abnormalities were seen in two AIDS patients and in two otherwise somatically healthy cases. Clinical data of the patients are given in Tables I and II. A group of 14 healthy and drug-free volunteers, aged 23-58 years (mean age 33), was used for comparative analyses.

CSF

CSF and blood samples were collected from patients and controls in the morning before breakfast. The subjects were instructed to rest, but they were not confined strictly to bed during the night before sampling. With the subject in a lateral recumbent position, the lumbar puncture was made between L IV and L V, and 22 ml of CSF was obtained from each person. In accordance with standardized procedures, CSF flow was divided into fractions for use in various analyses; concentrations of 5-HIAA and tryptophan were determined in the 13:th

ml fraction. Samples were frozen at -70°C immediately after collection and stored until analysed.

An ethylenediaminetetraacetic acid (EDTA) blood sample was drawn from each individual in connection with the CSF sampling. Immediately after sampling for duplicate determinations of tryptophan and 5-HT, 2×2 ml blood was transferred to new glass tubes containing 6 mg ascorbic acid. All samples were frozen and stored at -20°C until they were analysed. Platelets were counted in fresh blood from each sample for estimation of 5-HT content per platelet.

Analytical Techniques

The indole amine analyses in blood and CSF were performed by means of high-performance liquid chromatography (HPLC) technique with fluorescence detection. The Perkin-Elmer LS 4 detector was set to $\text{Ex} = 280$ nm and $\text{EM} = 345$ nm, with slits of 10 nm and 20 nm, respectively. The column was a $10 \mu\text{m}$ $\mu\text{Bondapack C}_{18}$ ($3.9 \text{ mm} \times 30 \text{ cm}$) from Waters Associated Inc, set at a flow rate of 1.5 ml/min. The mobile phase consisted of a 0.01 M citric acid monohydrate/tri-sodium citrate 2-hydrate buffer at $\text{pH} = 4.1$, and CH_2OH (92:8). Imme-

TABLE II. Pharmacological Treatment in 24 Patients With HIV Infection

Case number	Substances
1	Amphotericin B
2	Aciclovir + pyrimethaminum + calcii folinas + amphotericin B
3	Aciclovir + pyrimethaminum + calcii folinas + amphotericin B
4	No medication
5	Trimethoprim/sulfamethoxazolium + flunitrazepam
6	No medication
7	Dextropropoxiphenum + paracetamol/chlormezanolum + nitrazepam
8	No medication
9	Metronidazolium + paramomycinum
10	No medication
11	Thioridazine prescribed but probably not taken
12-16	No medication
17	Clóxacillinatrium + dextropropoxiphenum
18-22	No medication
23	Diazepam + alimemazinum
24	No medication

diately after thawing, the blood samples were precipitated with 200 μ l ascorbic acid (10%), 200 μ l EDTA (10%), and 8 ml perchloric acid (0.4 M). After centrifugation, 25 μ l of the clear supernatant was injected into the described HPLC system for assay of 5-HT and tryptophan. From each thawed CSF sample, 50 μ l was directly injected into the same HPLC system for assay of tryptophan and 5-HIAA (Larsson et al., 1988a).

HIV antibodies were tested by enzyme-linked immunosorbent assay (Behring and Wellcome) and confirmed by Western blot. Electroimmunoassay was used for determination of the serum concentrations of albumin (Laurell, 1972).

The number of T-helper-inducer cells ($CD4^+$) was measured by means of leu-3 monoclonal antibodies according to standard methods.

Statistics

Statistical evaluations of differences between groups of values were performed by means of a *t*-test.

RESULTS

Tryptophan in Blood and CSF

Blood and CSF concentrations of tryptophan in the HIV patients and healthy subjects are presented in Table III. Blood tryptophan levels below the lowest value in the group of healthy subjects were seen in seven asymptomatic patients, two patients with LAS, one with GB, and five with AIDS. CSF concentrations of tryptophan below the lowest value in the healthy group were found in 11 of the 14 asymptomatic patients, in both patients

with LAS, in the patient with ARC, and in all patients with AIDS (Fig. 1).

The tryptophan levels were significantly lower in the asymptomatic patients than in the healthy subjects ($P < 0.01$ for blood as well as for CSF concentrations) and still further reduced in the patients with AIDS ($P < 0.001$ for blood as well as for CSF concentrations (Fig. 1). The lowest blood tryptophan value, 2.8 μ g/ml, was seen in two patients suffering from dementia (cases 1 and 3).

The blood/CSF tryptophan concentration ratio in the healthy subjects ($n = 14$) varied between 14.6 and 23.0 (mean 18.0). Ratios above 23.0 were noted in one patient with AIDS and in five other cases. The range among the other 18 patients was 9.2–23.0 (mean 16.8).

5-HIAA in CSF

No significant change in the CSF 5-HIAA concentrations was observed among the HIV patients (Table III), but in two asymptomatic patients, one symptomatic patient, and one AIDS patient, i.e., in 17% of the cases, 5-HIAA concentrations were reduced as compared with healthy subjects.

Platelets and 5-HT

The number of platelets was significantly lower in patients with HIV infection than in the healthy subjects (Fig. 2, Table III). Five asymptomatic patients, one with GB, and two with AIDS had platelet counts below the lowest value in the control group.

Blood concentrations of 5-HT in the HIV patients were, in mean, 50% lower than in the healthy subjects

Table III. Laboratory Findings in Healthy Subjects and Patients With HIV Infection*

	Healthy subjects (n = 14)		HIV patients (n = 24)		Reduction in mean values (%)	Significance of difference
	Mean	Range	mean	range		
Number of platelets ($\times 10^9$ /liter)	238	140-375	184	32-287	23	$P < 0.02$
Blood concentration of 5-HT (ng/ml)	153	97-374	76	15-175	50	$P < 0.001$
Platelet-bound 5-HT (ng/ 10^9 platelets)	676	416-1257	430	64-1018	36	$P < 0.01$
Blood concentration of tryptophan (μ g/ml)	8.1	6.0-11.1	5.8	2.8-9.2	28	$P < 0.001$
CSF concentration of tryptophan (ng/ml)	445	371-513	310	30-531	30	$P < 0.001$
CSF concentration of 5-HIAA (ng/ml)	23	13-42	25	10-47		n.s.
Serum concentration of albumin (g/liter)	41.5	37-49	40.3	29-51	2.9	n.s.

*n.s. = not significant.

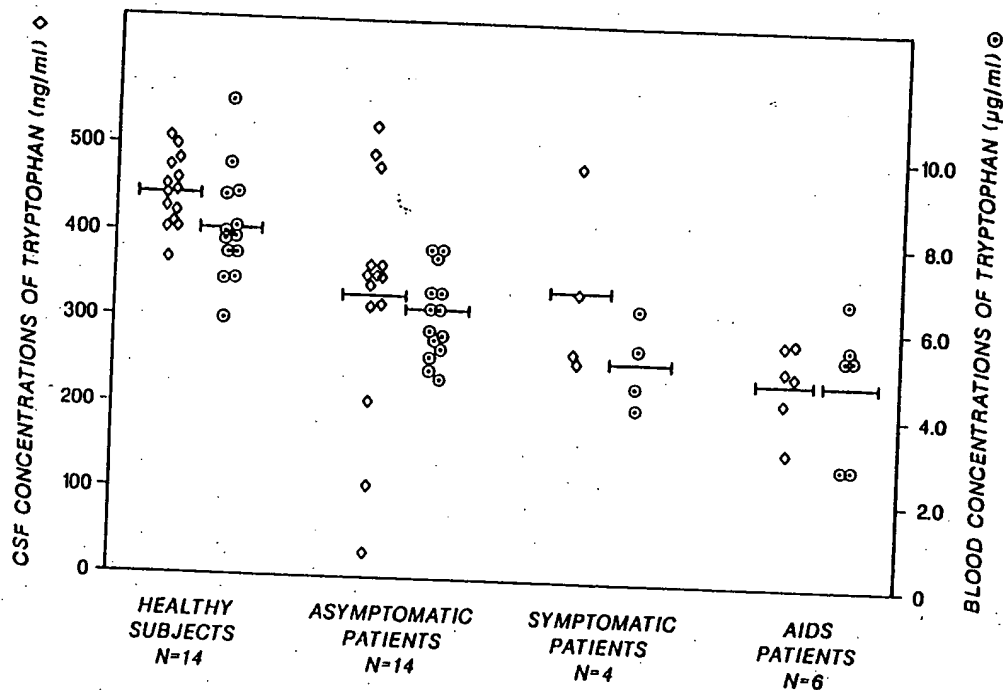


Fig. 1. Blood and CSF concentrations of tryptophan in healthy subjects and in patients with different degrees of HIV infection. Mean value is indicated in each group.

(Table III). The ratio between the blood concentrations of 5-HT and the number of platelets, i.e., the platelet content of 5-HT, was found to be reduced in the HIV patients (Table III). Levels of platelet-bound 5-HT below the lowest value in the control group were found in six asymptomatic patients, in two symptomatic patients (1 with LAS, 1 with GB), and in five of the six AIDS patients. The mean 5-HT content of the platelets was decreased with 30% in the asymptomatic patients ($P < 0.02$) and with 56% in the AIDS patients ($P < 0.01$), as compared with the corresponding value in the control group. This decrease in the mean platelet-bound 5-HT levels with increasing severity of HIV infection is illustrated in Figure 2.

Among the six patients with AIDS, the lowest lev-

els of 5-HT per 10^9 platelets were seen in the three patients who also had the highest number of platelets (Fig. 2). A normal number of platelets, in combination with decreased amounts of platelet-bound 5-HT, was also observed in one patient with LAS and in four asymptomatic patients. No similar finding was made in the group of healthy subjects.

CD4⁺ Cells and Tryptophan

The total number of CD4⁺ cells was decreased in those patients having a severe degree of HIV infection (Table I). A logarithmic regression model showed a positive correlation between the total number of helper cells (x) and the blood concentration of tryptophan (y) in the

creased severity of the infection. Also, the lowest tryptophan levels were found among the patients with AIDS.

The HIV patients also had reduced CSF concentrations of tryptophan as compared with healthy subjects. The blood/CSF concentration gradient of tryptophan, however, was essentially unchanged in all but two of the HIV patients (cases 11 and 22, who also had signs of organic brain disease (Table I)). Three other symptomatic patients and one AIDS patient had slightly elevated ratios. The fact that the ratios in most cases were unchanged indicated that the low CSF tryptophan concentrations might be due to low blood concentrations of the amino acid rather than to degradation inside the CNS. In addition, the majority of the HIV patients had CSF concentrations of 5-HIAA within the range of values among the healthy subjects, indicating a functional synthesis of the neurotransmitter. This finding does not, however, exclude possible changes in the release and turnover of the transmitter or in the postsynaptic receptor functions. Findings in the four patients with decreased CSF concentrations of 5-HIAA could indicate an affected metabolic pathway of serotonin in the CNS in these cases.

The number of platelets, as well as the concentrations of 5-HT in blood, was reduced in patients with HIV infection. Because the blood-5-HT to a great extent is bound to the platelets, which store the amine but do not synthesize it, concentrations of 5-HT were related to the number of platelets. Also, the platelet content of 5-HT was reduced in patients with HIV infection when compared with healthy subjects. The most pronounced reductions in platelet-bound 5-HT were seen in AIDS patients with a normal number of platelets. As the difference could not be ascribed to any known mechanism associated with the medication in the patient group (Table II), the low platelet content of 5-HT could be caused by defective platelets (Stricker et al., 1985; Beardsley et al., 1984), or they might result from a defective tryptophan metabolism.

ACKNOWLEDGMENTS

This study was supported by the Swedish Medical Research Council (grants number 5996 and 7746), by the Söderström-König Foundation, and by the University of Göteborg.

REFERENCES

- Åsjo B, Fenyö EM, Norkrans G, Hagberg L, Albert J (1986): Isolation of human immunodeficiency virus from cerebrospinal fluid of an antibody-positive carrier without neurological symptoms. *Lancet* 1:1276-1277.
- Beardsley DS, Spiegel JE, Jacobs MM, Handin RI, Lux SE (1984): Platelet membrane glycoprotein IIIa contains target antigens that bind anti-platelet antibodies in immune thrombocytopenias. *J Clin Invest* 74:1701-1707.
- Chiodi F, Sönnnerborg A, Albert J, et al. (1988): Human immunodeficiency virus infection of the brain. *J Neurol Sci* 85:245-257.
- Ho D, Rota TR, Schooley RT, Kaplan JC, Allan JD, Groopman JE, et al. (1985): Isolation of HTLV-III from cerebrospinal fluid and neural tissues of patients with neurologic syndromes related to the acquired immunodeficiency syndrome. *N Engl J Med* 313:1493-1497.
- Ho D, Pomerantz RJ, Kaplan JC (1987): Pathogenesis of infection with human immunodeficiency virus. *N Engl J Med* 317:278-284.
- Koenig S, Gendelman HE, Orenstein JM, Dal Canto MC, Pezeshekpour GH, Youngbluth M, et al. (1986): Detection of AIDS virus in macrophages in brain tissue from AIDS patients with encephalography. *Science* 233:1089-1093.
- Larsson M, Forsman A, Hallgren J (1988a): HPLC assays of 5-HIAA and tryptophan in cerebrospinal fluid and of 5-HT and tryptophan in blood: a methodological study with clinical applications. *Methods Find Exp Clin Pharmacol* 10:453-460.
- Larsson M, Forsman A, Hagberg L, Norkrans G (1988b): HIV and biochemical findings in blood and CSF. IV International Conference on AIDS (Stockholm) 1:B-2168.
- Laurell CB (1972): Electroimmunoassay. *Scand J Clin Lab Invest* 29(suppl 124):21-37.
- Navia BA, Jordan BD, Price RW (1986): The AIDS dementia complex. I: Clinical features. *Ann Neurol* 19:517-524.
- Nelson JA, Reynolds-Kohler C, Margaretten W, Wiley CA, Reese C, Levy JA (1988): Human immunodeficiency virus detected in bowel epithelium from patients with gastrointestinal symptoms. *Lancet* 1:259-260.
- Serwadda D, Mugerwa RD, Sewankambo N, et al. (1985): Slim disease: a new disease in Uganda and its association with HTLV-III infection. *Lancet* 2:849-852.
- Stricker RB, Abrams DI, Corash L, Shuman MA (1985): Target platelet antigen in homosexual men with immune thrombocytopenia. *N Engl J Med* 313:1375-1385.
- Werner E, Fuchs D, Hausen A, Jaeger H, Reibnegger G, et al. (1988): Tryptophan degradation in patients infected by human immunodeficiency virus. *Biol Chem Hoppe Seyler* 369:337-340.
- Wiley CA, Shrier RD, Nelson JA, Lampert PW, Oldstone MB (1986): Cellular localization of human immunodeficiency virus infection within the brains of acquired immunodeficiency syndrome patients. *Proc Natl Acad Sci* 83:7089-7093.

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A

Decreased Serum Tryptophan in Patients with HIV-1 Infection Correlates with Increased Serum Neopterin and with Neurologic/Psychiatric Symptoms

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Summary: We investigated serum neopterin, tryptophan, and kynurenine concentrations in 23 HIV-1 seropositive patients (Walter Reed Stage 4-6). Ten patients presented with polyneuropathy and three with dementia, one of the patients with dementia also had polyneuropathy and dementia. We found significant associations between lower tryptophan concentrations and neurologic/psychiatric symptoms. The negative correlation of tryptophan with kynurenine and neopterin concentrations indicates activity of indoleamine 2,3-dioxygenase (IDO) in patients. IDO can be induced by cytokines such as interferon- γ and therefore low tryptophan levels may result from chronic immune stimulation in HIV-1 seropositives. **Key Words:** Serum tryptophan—Neopterin—Dementia—Polyneuropathy.

Neurologic dysfunction and destruction are frequent complications of human immunodeficiency virus type 1 (HIV-1) infection. However, the mechanisms by which symptoms such as dementia or polyneuropathy are mediated have not been elucidated (1). Disturbance of tryptophan metabolism was suggested to be involved (2) and reduced tryptophan together with increased kynurenine as well as reduced serotonin concentrations were reported to occur frequently in the sera of patients with advanced HIV-1 infection (3,4). We were interested in investigating a possible association between disturbed tryptophan metabolism, chronic immune stimulation, and neurologic/psychiatric symptoms in patients with HIV-1 infection.

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We investigated serum tryptophan, kynurenine, and neopterin concentrations by high-performance liquid chromatography (3) in 23 patients (all male, 12 homosexuals, 6 intravenous drug abusers, 5 homosexuals with intravenous drug abuse; median age 35.0 years, range 22-54) with proven HIV-1 infection (Abbott enzyme-linked immunosorbent assay positive, confirmed by Western blot). Four patients were classified as Walter Reed Stage (WR) 4, 9 were WR5, and 10 were WR6. Ten patients (three with WR4, three with WR5, four with WR6) presented with polyneuropathy. One of the WR6 patients with polyneuropathy also had dementia as did two further WR6 patients without polyneuropathy.

Polyneuropathy was diagnosed clinically and confirmed by electroneurophysiological examination, which was performed in each case of patient complaints (cramps, dysesthesia) or findings (disturbances of motor and/or sensory functions afflicting the extremities, loss of tendon reflexes) sugges-

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tive of affection of peripheral nerves. To test for neurologic dysfunction and destruction we quantified ventricular enlargement as measured by the ventricle-brain ratio in computerized cranial tomography (5).

The score of a structured interview and brief neuropsychological testing was used for the diagnosis of dementia. The tests rely on DSM-III-R and ICD-10 algorithms including the Mini Mental State (6) and allow a detailed measurement of even low levels of cognitive impairment. The test provides quantification of severity grading of cognitive dysfunctions that are reflected by lower scores (highest score is 55).

Statistical comparisons were performed applying Student's *t* test. For correlation analyses, Spearman's rank correlation coefficients (r_s) were used.

Serum tryptophan levels were lower in our patients with HIV-1 infection compared with healthy blood donors (Table 1), which confirms earlier results (3). Moreover, patients with polyneuropathy and/or dementia had lower serum tryptophan levels compared with those without such symptoms (Table 1). Three patients with dementia had the lowest tryptophan levels (31.2, 34.4, and 34.8 $\mu\text{mol/L}$). In contrast, serum kynurenine and neopterin levels were higher in patients compared with healthy controls. Neopterin and kynurenine levels did not differ in patients with additional symptoms compared with those without (Table 1).

Tryptophan and neopterin levels were negatively correlated ($r_s = -0.608$, $p < 0.01$). In addition, tryptophan and neopterin concentrations were correlated with ventricle brain ratio (neopterin: $r_s = 0.588$, $p < 0.01$; tryptophan: $r_s = -0.444$, $p < 0.05$) as well as with the dementia score (neopterin: $r_s = -0.461$, $p < 0.05$; tryptophan: $r_s = 0.447$, $p < 0.05$).

The significant correlations with neopterin may indicate that chronic immune activation is involved in the disturbed tryptophan metabolism and the pathogenesis of neurologic/psychiatric symptoms. Large amounts of neopterin are produced by human monocytes/macrophages on stimulation with interferon- γ (7), and other cytokines such as tumor necrosis factor α can amplify its action (8). Recently, interferon- γ and tumor necrosis factor α were reported to be increased in sera of patients with HIV-1 infection (9,10). In vitro data showed that interferon- γ is also able to induce indoleamine 2,3-dioxygenase (IDO), which degrades tryptophan to kynurenine (8). In our patients with HIV-1 infection, increased activity of IDO is indicated by increased serum kynurenine levels in patients with decreased tryptophan. A positive correlation existed between kynurenine and neopterin levels ($r_s = 0.419$, $p < 0.05$), which supports the concept that chronic immune activation and increased degradation of tryptophan, rather than reduced dietary intake, may be the reason for reduced tryptophan levels. The independence from the nutritional status is further supported by the finding that serum albumin did not differ between patients with and without dementia and/or polyneuropathy. In addition, no correlation between serum albumin and any other variable of the study was found (data not shown).

Kynurenine levels were high in HIV-1 seropositive patients but no further increase was seen in the patients with dementia and/or polyneuropathy compared with those without. This finding agrees with in vitro observations that IDO is the only enzyme within the metabolic pathway of tryptophan degradation that is activated by interferon- γ (8). Other enzymes downstream in the degradation pathway are constitutively present in cells. Thus, kynurenine formed by IDO is likely to be further metabolized

TABLE 1. Tryptophan, kynurenine, and neopterin levels (mean \pm SD) in HIV-1 seropositive patients with and without dementia and/or polyneuropathy (for comparison levels on HIV-1 seronegative blood donors are shown)

	Tryptophan ($\mu\text{mol/L}$)	Kynurenine ($\mu\text{mol/L}$)	Neopterin (nmol/L)
HIV-1 seropositive patients with dementia and/or polyneuropathy (n = 12)	48.8 \pm 13.4	3.5 \pm 0.78	30.8 \pm 19.8
	t = 3.59 p < 0.01	t = 0.25 NS ^a	t = 1.90 NS ^a
HIV-1 seropositive patients without dementia and/or polyneuropathy (n = 11)	70.5 \pm 15.6	3.6 \pm 1.14	18.6 \pm 8.2
	t = 2.53 p < 0.05	t = 3.13 p < 0.01	t = 5.61 p < 0.01
HIV-1 seronegative blood donors (n = 11)	91.1 \pm 22.0	2.3 \pm 0.77	4.5 \pm 1.5

^a Student's *t* test; NS, not significant.

downstream in the degradation pathway of tryptophan to form 3-hydroxykynurenine, 3-hydroxyanthranilic acid, and quinolinic acid. Quinolinic acid has already been demonstrated to be increased in patients with ARC and AIDS (11).

Activation of IDO can also be represented by the ratio of the product concentration (kynurenine) versus the substrate concentration (tryptophan). A highly significant positive correlation between the kynurenine/tryptophan quotient and neopterin (Fig. 1) indicates that both events, neopterin release and activation of IDO, can be referred to the specific activity of cytokines, such as interferon- γ and tumor necrosis factor α .

Our data show that low serum tryptophan is associated with neurologic dysfunction in HIV-1 infection. Decreased serum tryptophan as well as increased neopterin are associated with the degree of neurologic disturbances in patients with HIV-1 infection as expressed by correlations between the markers and computed tomography results as well as dementia scores. Reduced tryptophan levels are very likely to result from persistent immune activation. This is underlined by the strong negative correlation between neopterin and tryptophan concentrations. Moreover, the best correlation was found between neopterin levels and the ratio of kynurenine to tryptophan, representing an estimate of IDO activity, an enzyme that is inducible by cytokines.

In summary, all biochemical changes discussed may result from continuous release of cytokines such as interferon- γ and tumor necrosis factor α .

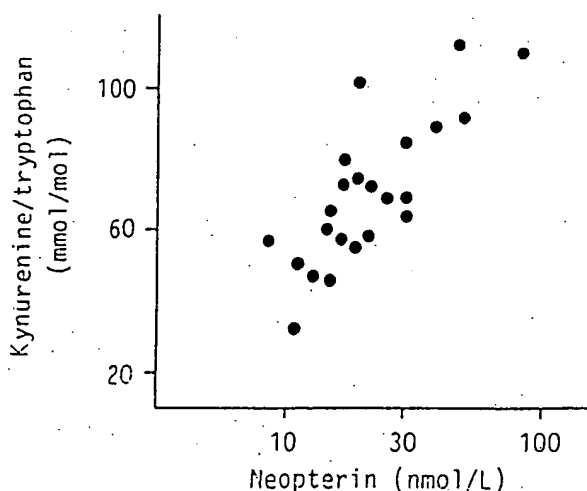


FIG. 1. Correlation between serum neopterin levels and the ratio between kynurenine and tryptophan concentrations as an estimate for indoleamine (2,3)-dioxygenase ($r_s = 0.826$, $p < 0.01$; Spearman rank correlation coefficient).

The background of chronic immune activation may be multifactorial. Besides HIV-1, other infectious agents could be important. However, highly increased neopterin concentrations were not only found in HIV-seropositive individuals in high-risk groups but also in transfusion recipients (12).

Increased neopterin concentrations can be observed early during the course of HIV-1 infection (7,13), and neopterin levels further increase during the course of the infection. However, only chronic and high-level immune activation appears to cause degradation of tryptophan in a range that is relevant compared with the dietary intake.

Low tryptophan concentrations may explain decreased serum serotonin levels that were reported to occur frequently among HIV-1 seropositive patients (4). We expect that similar associations between active tryptophan metabolism and chronic immune activation can also be established intrathecally. Reduced tryptophan and serotonin levels (14) and increased neopterin levels (13,15) in the cerebrospinal fluid have already been shown to be associated with dementia and/or polyneuropathy in patients with HIV-1 infection.

REFERENCES

1. Ho DD. The acquired immunodeficiency syndrome (AIDS) dementia complex. *Ann Intern Med* 1989;111:400-10.
2. Fuchs D, Werner ER, Dierich MP, Wachter H. Cellular immune activation in the brain and human immunodeficiency virus infection. *Ann Neurol* 1988;24:289.
3. Werner ER, Fuchs D, Hausen A, et al. Tryptophan degradation in patients infected by human immunodeficiency virus. *Biol Chem Hoppe-Seyler* 1988;369:337-40.
4. Launay JM, Copel L, Callebort J, et al. Decreased whole blood 5-hydroxytryptamine (serotonin) in AIDS patients. *J Acq Immune Synd* 1988;1:324-5.
5. Synek V, Reuben JR. The ventricular brain ratio using planimetric measurement of EMI scans. *Br J Radiol* 1976;49:233-7.
6. Folstein NF, Folstein SE, McHugh PR. "Mini Mental State"—a practical method for grading the cognitive state of patients for the clinician. *J Psychiatr Res* 1975;12:189-98.
7. Fuchs D, Hausen A, Reibnegger G, Werner ER, Dierich MP, Wachter H. Neopterin as a marker for activated cell-mediated immunity: application in HIV infection. *Immunol Today* 1988;9:150-5.
8. Werner-Felmayer G, Werner ER, Fuchs D, et al. Tumour necrosis factor- α and lipopolysaccharide enhance interferon-induced tryptophan degradation and pteridine synthesis in human cells. *Biol Chem Hoppe-Seyler* 1989;370:1063-9.
9. Fuchs D, Hausen A, Reibnegger G, et al. Interferon gamma concentrations are increased in sera from individuals infected with human immunodeficiency virus type 1. *J Acq Immune Def Synd* 1989;2:158-62.
10. Laehdevirta J, Maury CPJ, Teppo AM, Repo H. Elevated

- levels of circulating cachectin/tumor necrosis factor in patients with acquired immunodeficiency syndrome. *Am J Med* 1988;85:289-91.
11. Heyes MP, Rubinoco D, Lane C, Markey SP, Price R, Salazar A. Cerebrospinal fluid quinolinic acid concentrations are increased in acquired immunodeficiency syndrome. *Ann Neurol* 1989;26:275-7.
 12. Hollan SR, Ujhelyi E, Paloczi K, et al. Results of longitudinal immunological surveillance of individuals directly or indirectly infected by a single HIV seropositive donor. *Transfusion Sci* (in press).
 13. Sönnnerborg AB, von Stedingk LU, Hansson LO, Strannegard OO. Elevated neopterin and beta-2-microglobulin levels in blood and cerebrospinal fluid occur early in HIV-1-infection. *AIDS* 1989;3:277-84.
 14. Larsson M, Hagberg L, Norkrans G, Forsman A. Indoleamine deficiency in blood and cerebrospinal fluid from patients with human immunodeficiency virus infection. *J Neurosci Res* 1989;23:441-6.
 15. Fuchs D, Chiodi F, Albert J, et al. Neopterin concentrations in cerebrospinal fluid and serum of individuals infected with HIV-1. *AIDS* 1989;3:285-8.

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Immune Activation and Decreased Tryptophan in Patients with HIV-1 Infection

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ABSTRACT

We compared tryptophan, neopterin, and interferon- γ (IFN- γ) concentrations in serum and cerebrospinal fluid (CSF) of 22 patients with human immunodeficiency virus type 1 (HIV-1) infection. Tryptophan levels were found to be decreased in CSF and serum of patients whereas neopterin levels in CSF and serum and serum IFN- γ concentrations were increased compared to healthy HIV-1 seronegatives. Tryptophan concentrations correlated negatively to neopterin concentrations, and serum neopterin concentrations correlated positively to IFN- γ concentrations. Thus, decrease of tryptophan levels is associated with chronic immune stimulation in patients with HIV-1 infection. From the data it appears that reduced tryptophan in patients may result from induction of indoleamine (2,3)-dioxygenase by IFN- γ .

INTRODUCTION

PATIENTS WHO ARE INFECTED with human immunodeficiency virus type 1 (HIV-1) frequently present with neurologic and psychiatric symptoms.⁽¹⁾ Impaired tryptophan metabolism and reduced availability of 5-hydroxytryptamine (serotonin) could be involved.⁽²⁻⁴⁾ Reduced concentrations of tryptophan were reported in serum and cerebrospinal fluid (CSF) of patients.^(2,3) Decreased tryptophan could result from increased activity of indoleamine (2,3)-dioxygenase (IDO), which is induced by interferon- γ (IFN- γ) and degrades tryptophan to form kynurenine, which then is further metabolized.⁽⁵⁾ Increased concentrations of neopterin in patients with HIV-1 infection⁽⁶⁻⁸⁾ reflect chronic immune stimulation. Large quantities of neopterin are released by human macrophages on stimulation with IFN- γ *in vitro*.⁽⁹⁾ Release of neopterin by stimulated macrophages is paralleled by significant degradation of tryptophan.⁽¹⁰⁾ Recently, increased concentrations of IFN- γ were reported in serum of HIV-1 seropositives.⁽¹¹⁾

In this study, we compared tryptophan concentrations in serum and CSF of patients with HIV-1 infection to the immune activation markers neopterin and IFN- γ . Neopterin and tryptophan data stem from two earlier

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studies, which reported decreased tryptophan⁽³⁾ and increased neopterin⁽⁶⁾ levels in serum and CSF of a larger cohort of HIV-1 seropositives.

METHODS

Neopterin and tryptophan levels were measured in serum and CSF samples from 22 patients with established HIV-1 infection (ELISA-positive, confirmed by Western blot). Fourteen patients were asymptomatic, four had persistent generalized lymphadenopathy (LAS), and four had AIDS at time of enrollment (Table 1). One LAS patient was treated with metronidazolum and paramomycinum, and the

TABLE 1. PATIENT CHARACTERISTICS AND LABORATORY RESULTS

Case ^a	Clinical status ^b	Age (yrs)	Tryptophan (umole/liter)		Neopterin (nmole/liter)		IFN- γ (U/liter)	
			Serum	CSF	Serum	CSF	Serum	CSF
1	AS	28	45.1	2.36	12.4	2.0	— ^c	—
2	AS	37	36.7	1.68	10.6	1.2	—	—
3	AS	25	30.9	1.76	19.6	3.3	34	206
4	AS	25	28.4	1.78	4.9	1.2	21	189
5	AS	31	37.7	1.79	5.8	1.3	78	137
6	AS	28	24.0	2.60	11.2	2.4	77	135
7	AS	47	25.5	2.42	9.5	2.7	37	140
8	AS	39	23.0	0.15	38.4	27.1	99	189
9	AS	21	28.9	1.02	18.3	9.5	50	53
10	AS	25	30.9	1.88	10.6	10.7	50	78
11	AS	65	25.5	1.63	12.8	7.2	26	166
12	AS	48	19.6	1.58	19.8	16.9	240	128
13	AS	26	41.6	1.21	9.9	12.3	51	123
14	AS	51	26.9	0.57	11.4	10.1	44	95
15	LAS	33	32.8	1.43	13.6	4.5	—	—
16	LAS	27	31.3	0.97	9.8	8.3	20	134
17	LAS	49	27.4	1.24	31.4	4.5	394	255
18	LAS	41	31.3	1.27	12.2	4.8	44	124
19	AIDS	40	41.1	1.38	21.6	8.4	—	—
20	AIDS	37	13.7	1.23	126.6	72.7	321	203
21	AIDS	31	26.4	1.39	30.5	12.4	1055	188
22	AIDS	46	26.4	1.22	37.4	23.2	229	71
Mean		36	29.8	1.48	21.7	11.2	159	143
SD		±11	±7.4	±0.57	±25.3	±15.4	±250	±53
HIV seronegative controls ^(3,6,11)								
n			14	14	359	19	79	—
Mean			39.7	2.18	5.34	1.60	33	—
SD			±8.8	±0.19	±2.70	±0.60	±15	—

^aAll cases are male homosexuals except case 2 (female blood transfusion recipient), case 22 (female with heterosexual transmission), and case 14 (male intravenous drug user).

^bAS, Asymptomatic; LAS, lymphadenopathy syndrome. All AIDS patients had at least one episode of *Pneumocystis carinii* pneumonia; case 19 had additional Kaposi's sarcoma.

^cNot done.

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AIDS patients were treated with amphotericin B₄ ($n = 1$), aciclovir + pyrimethaminum + calcii folinas + amphotericin B ($n = 2$), or trimethoprimum/sulfamethoxazolium + flunitrazepanum ($n = 11$). All others were free of therapy at the time of enrollment.⁽³⁾

Tryptophan concentrations in serum and CSF were determined by high-pressure liquid chromatography.⁽³⁾ Neopterin levels in serum and CSF were measured by radioimmunoassay (RIAid, Fa. Henning-Berlin, Berlin, FRG) as previously described.⁽⁶⁾ IFN- γ was measured in 18 serum and 18 CSF samples by radioimmunoassay (Centocor Inc., Malvern, PA). The sensitivity of this test was increased to 18 U/liter by increasing the incubation time as described.⁽¹¹⁾ The test results were compared to HIV-seronegative healthy controls, who have been examined in our laboratories during the underlying primary studies^(3,6) and in an earlier study.⁽¹¹⁾

Comparison of grouped data was done by Student's t test and results were confirmed by nonparametric tests (not shown in detail). To test for associations between variables we used Spearman's rank correlation coefficients r_s .

RESULTS

Patients with HIV-1 infection had decreased tryptophan ($p < 0.01$ Student's t test) and increased neopterin concentrations ($p < 0.01$) in serum and CSF compared to HIV-1 seronegative controls (Table 1). Circulating IFN- γ in serum was increased ($p < 0.01$). IFN- γ was also detectable in CSF. Treatment status was not associated with any of the parameters in the group of patients (not shown).

Negative correlations existed between neopterin and tryptophan concentrations in both serum ($r_s = -0.42$, $p = 0.05$; Fig. 1) and CSF ($r_s = -0.66$, $p < 0.01$). Serum IFN- γ correlated positively with serum neopterin ($r_s = 0.65$, $p < 0.01$) and negatively but not significantly with serum tryptophan levels ($r_s = -0.39$, n.s.). CSF concentrations of IFN- γ did neither correlate with neopterin nor with tryptophan concentrations in the CSF ($r_s = -0.22$ and 0.20 ; n.s.).

To explore a possible statistical bias caused by the four AIDS patients, we reevaluated the correlation analyses considering exclusively asymptomatic individuals and patients with lymphadenopathy syndrome. However, the results of the statistics and the interpretation of the data did not change.

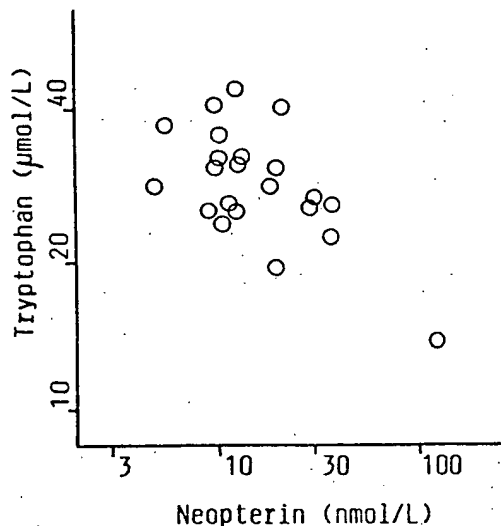


FIG. 1. Correlation between serum neopterin and tryptophan levels in patients with HIV-1 infection (note the double-logarithmic scale). The data suggest a reciprocal relationship, which is significant (tryptophan⁻¹ = 0.0285 + 3.41 × 10⁻⁴ × neopterin; $r = 0.802$, $p < 0.0001$).

DISCUSSION

HIV-1 infection is associated with increased neopterin⁽⁶⁻⁹⁾ and decreased tryptophan^(2,3) concentrations in serum and CSF. Increased levels of circulating IFN- γ have also been reported.⁽¹¹⁾ The results of the present evaluation confirm these earlier findings. The three variables correlated to a variable degree with the severity of symptoms in the patients. However, the number of samples in the groups with different clinical presentation is too small for meaningful statistical evaluation.

In our patients with HIV-1 infection, we found a positive correlation between serum IFN- γ and neopterin concentrations. In addition, we found that serum tryptophan levels correlate negatively to neopterin. The data agree with the hypothesis that endogenous release of IFN- γ may be the reason for these metabolic changes in patients. Decreased tryptophan appears to be associated with chronic immune activation and may result from increased activity of IDO. IDO and release of neopterin can be induced by IFN- γ .^(5,9,10)

The correlation between serum tryptophan and IFN- γ was negative, but was just below statistical significance. The small number of patients in this study could be the reason for the lack of significance. Further studies may help to clarify the possible presence of a correlation between tryptophan and IFN- γ data. Obviously, other cytokines such as IFN- α and tumor necrosis factor- α (TNF- α) can modulate IFN action and hence contribute to additional changes in neopterin and tryptophan concentrations.^(9,12) In addition, IFN- γ may bind to receptors on cells,⁽¹³⁾ and so free IFN- γ would not reflect its biological activity. This may explain in part why the direct correlation between neopterin and tryptophan was better than that to IFN- γ .

Also CSF neopterin and tryptophan concentrations correlated negatively and IFN- γ could be detected in CSF. Mean IFN- γ levels were similar in serum and CSF samples but many patients had CSF levels higher than those in serum. However, the levels showed only small variation. No correlation existed between CSF IFN- γ and neopterin or tryptophan concentrations in the CSF. Unfortunately, no CSF IFN- γ reference levels of healthy controls were available. Further studies are needed to demonstrate a possible relevance of intrathecal production of IFN- γ in patients with HIV-1 infection and the reliability of the test used in CSF samples. However, intrathecal production of the immune activation markers β_2 -microglobulin and neopterin has already been demonstrated in patients with HIV-1 infection.^(6,8)

The relevance of IDO activation in HIV-1 seropositive patients is further supported by earlier data showing increased levels of tryptophan metabolites such as kynurenine in serum⁽²⁾ and quinolinic acid in the CSF,⁽¹⁴⁾ confirming that decreased tryptophan is not simply due to reduced dietary intake.^(3,15)

Notably, disturbances of tryptophan metabolism are seen in early stages of HIV-1 infection and independently from neurologic or psychiatric abnormalities of patients. However, a significant correlation between decreased tryptophan and neuropsychological disturbances such as dementia and polyneuropathy in patients with HIV-1 infection was demonstrated in earlier studies.^(3,15) In the small group of patients in our study, we were not able to examine this relationship. It remains to be shown whether neurotoxic tryptophan metabolites⁽¹⁶⁾ or altered availability of serotonin,^(3,4) or both effects together, contribute to neuropsychologic deterioration in patients. Both metabolic abnormalities, however, may result from chronic challenge of cell-mediated immunity and concomitant release of cytokines in patients with long-lasting illness such as HIV-1 infection.

ACKNOWLEDGMENT

This work was financially supported by the Österreichisches Bundesministerium für Wissenschaft und Forschung, Sektion Forschung, and Swedish Medical Research Council grants nos. 7746 and 8772.

REFERENCES

1. JANSSEN, R.S., SAYKIN, A.J., CANNON, L., CAMPBELL, J., PINSKY, P.F., HESSOL, N.A., O'MALLEY, P.M., LIFSON, A.R., DOLL, L.S., RUTHERFORD, G.W., and KAPLAN, J.E. (1989). Neurological and neuropsychological manifestations of HIV-1 infection: association with AIDS-related complex but not asymptomatic HIV-1 infection. *Ann. Neurol.* 26, 592-600.

TRYPTOPHAN AND HIV-1 INFECTION

2. WERNER, E.R., FUCHS, D., HAUSEN, A., JÄGER, H., REIBNEGGER, G., WERNER-FELMAYER, G., DIERICH, M.P., and WACHTER, H. (1988). Tryptophan degradation in patients infected by human immunodeficiency virus. *Biol. Chem. Hoppe Seyler* **369**, 337-340.
3. LARSSON, M., HAGBERG, L., NORKRANS, G., and FORSMAN, A. (1989). Indoleamine deficiency in blood and cerebrospinal fluid from patients with human immunodeficiency virus infection. *J. Neurosci. Res.* **23**, 441-446.
4. LAUNAY, J.M., COPEL, L., CALLEBERT, J., CORVAIA, N., LEPAGE, E., BRICAIRE, F., SAAL, F., and PERIES, J. (1988). Decreased whole blood 5-hydroxy-tryptamine (serotonin) in AIDS patients. *J. Acquired Immune Defic. Syndr.* **1**, 324-325.
5. BYRNE, G., LEHMANN, L.K., KIRSCHBAUM, J.G., BORDEN, E.C., LEE, C.M., and BROWN, R.R. (1986). Induction of tryptophan degradation *in vitro* and *in vivo*: A gamma-interferon stimulated activity. *J. Interferon Res.* **6**, 389-398.
6. FUCHS, D., CHIODI, F., ALBERT, J., ASJÖ, B., HAGBERG, L., HAUSEN, A., NORKRANS, G., REIBNEGGER, G., WERNER, E.R., and WACHTER, H. (1989). Neopterin concentrations in cerebrospinal fluid and serum of individuals infected with HIV-1. *AIDS* **3**, 285-288.
7. FAHEY, J.L., TAYLOR, J.M.G., DETELS, R., HOFMANN, B., NISHANIAN, P., and GIORGI, J.V. (1990). The prognostic value of cellular and serologic markers in infection with human immunodeficiency virus type 1. *N. Engl. J. Med.* **322**, 166-172.
8. SÖNNERBORG, A.B., VON STEDINGK, L.V., HANSSON, L.O., and STRANNEGARD, O.O. (1989). Elevated neopterin and beta₂-microglobulin levels in blood and cerebrospinal fluid occur early in HIV-1 infection. *AIDS* **3**, 277-284.
9. FUCHS, D., HAUSEN, A., REIBNEGGER, G., WERNER, E.R., DIERICH, M.P., and WACHTER, H. (1988). Neopterin as a marker for activated cell-mediated immunity: Application in HIV-infection. *Immunol. Today* **9**, 150-155.
10. WERNER, E.R., BITTERLICH, G., FUCHS, D., HAUSEN, A., REIBNEGGER, G., SZABO, G., DIERICH, M.P., and WACHTER, H. (1987). Human macrophages degrade tryptophan upon induction by interferon-gamma. *Life Sci.* **41**, 273-280.
11. FUCHS, D., HAUSEN, A., REIBNEGGER, G., WERNER, E.R., WERNER-FELMAYER, G., DIERICH, M.P., and WACHTER, H. (1989). Interferon-gamma concentrations are increased in sera from individuals with human immunodeficiency virus type 1 infection. *J. Acquired Immune Defic. Syndr.* **2**, 158-162.
12. WERNER-FELMAYER, G., WERNER, E.R., FUCHS, D., HAUSEN, A., REIBNEGGER, G., and WACHTER, H. (1989). Tumour necrosis factor alpha and lipopolysaccharide enhance interferon induced tryptophan degradation and pteridine synthesis in human cells. *Biol. Chem. Hoppe Seyler* **370**, 1063-1069.
13. CARUSO, A., BONFATI, C., COLOMBRITA, D., DE FRANCESCO, M., DE RANGO, C., FORESTI, I., GARGIULO, F., GONZALES, R., GRIBAUDO, G., LANDOLFO, S., MANCA, N., MANNI, M., PIRALI, F., POLLARA, P., RAVIZZOLA, G., SCURA, G., TERLENGHI, L., VIANI, E., and TURANO, A. (1990). Natural antibodies to IFN-gamma in man and their increase during viral infection. *J. Immunol.* **144**, 685-690.
14. HEYES, M.P., RUBINOCO, D., LANE, C., MARKEY, S.P., PRICE, R., and SALAZAR, A. (1989). Cerebrospinal fluid quinolinic acid concentrations are increased in acquired immune deficiency syndrome. *Ann. Neurol.* **26**, 275-277.
15. FUCHS, D., MÖLLER, A.A., REIBNEGGER, G., STÖCKLE, E., WERNER, E.R., and WACHTER, H. (1990). Decreased serum tryptophan in patients with HIV-1 infection correlate with increased serum neopterin and with neurologic/psychiatric symptoms. *J. Acquired Immune Defic. Syndr.* **3**, 873-876.
16. SCHWARCZ, R., FOSTER, A.C., FRENCH, E.D., WHETSELL, W.O., and KÖHLER, C. (1984). Excitotoxic models for neurodegenerative disorders. *Life Sci.* **35**, 19-31.

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Received 27 March 1990/Accepted 5 July 1990

1991
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Increased endogenous interferon-gamma and neopterin correlate with increased degradation of tryptophan in human immunodeficiency virus type 1 infection

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(Received 4 February 1991; accepted 13 February 1991)

1. Summary

Reduced tryptophan and increased kynurenine concentrations have been reported in patients with human immunodeficiency virus type 1 (HIV-1) infection. From in vitro data it appears that activated indoleamine 2,3-dioxygenase (IDO) is involved in this metabolic change. IDO is inducible by interferon-(IFN)- γ . We compared serum concentrations of IFN- γ and neopterin (the biosynthesis of which is also inducible by IFN- γ) with serum tryptophan and kynurenine of 42 patients with HIV-1 infection.

IFN- γ , neopterin and kynurenine levels were significantly increased compared to HIV-1 seronegative controls whereas tryptophan was significantly decreased. Various significant correlations were found between tryptophan, kynurenine, IFN- γ and neopterin concentrations. Highest degree of correlation was found between neopterin, IFN- γ and the kynurenine per tryptophan quotient which is the ratio between the product and the substrate concentration of IDO.

The data indicate that decreased tryptophan in HIV-1 seropositives may result from chronic im-

mune activation and can be referred to increased activation of IDO.

2. Introduction

Progressive infection with HIV-1 is associated with substantial loss of immune function. Diminished capacity of immune cells to respond to infectious agents renders patients susceptible to severe secondary infections, which finally cause the acquired immune deficiency syndrome (AIDS) and death [1]. Despite this, patients with HIV-1 infection frequently show multiple signs of chronic immune activation such as the presence of circulating immune complexes [2], of acid-labile interferon-(IFN)- α [3], of a soluble form of interleukin-2 (IL-2) receptor [4, 5], and of increased neopterin concentrations in body fluids [5-7]. Also, circulating IFN- γ is increased in patients with HIV-1 infection compared to healthy HIV-1 seronegative controls [8]. A significant positive correlation between IFN- γ and neopterin concentrations was found in patients [8].

We described reduced concentrations of tryptophan and increased kynurenine levels in patients with human immunodeficiency virus type 1 (HIV-1) infection [9]. It appears possible that activated indoleamine 2,3-dioxygenase (IDO) is involved in this metabolic change. IDO cleaves tryptophan to form N-formylkynurenine, and the enzyme is inducible by IFN- γ [10].

We were interested, whether an association existed

Key words: HIV-1; indoleamine 2,3-dioxygenase; Interferon-gamma; Neopterin; Tryptophan

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EXHIBIT B

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between endogenous release of IFN- γ and changes of tryptophan metabolism in HIV-1 seropositives.

In this study, we compared IFN- γ and neopterin concentrations with tryptophan and kynurenine changes in 42 patients with HIV-1 infection.

3. Patients and Methods

Forty-two HIV-1 antibody-seropositives were included in this study (40 males, 2 females). They were aged 21–54 years (median 37.5 years). Twenty-eight were homosexuals, 6 intravenous drug users, 5 had both risk factors of HIV-1 exposure; for 3 patients, no apparent risk criteria could be established. According to the Walter Reed staging classification system (WR), 6 patients were WR2, 2 were WR3, 3 were WR4, 9 were WR5 and 21 were WR6. For one patient no CD4⁺ T cell counts were available. He did not suffer from AIDS. When comparing laboratory results of patients at different WR stages, the patient was included in group WR2–4. Sixteen patients (all WR5 or 6) were under regular treatment with Zidovudine (azidothymidine, AZT).

IFN- γ concentrations were quantified by radioimmunoassay (Centocor, Malvern, PA, U.S.A.). The sensitivity of the test was enhanced by employing an optimized assay procedure allowing the detection of IFN- γ in sera with sufficient sensitivity to define normal range of healthy HIV-1 seronegative controls [11]. Beads with monoclonal anti-human IFN- γ antibody were incubated with 200 μ l serum at room temperature for 16 h. Then the beads were washed with 3 ml of distilled water and incubated for a further 16 h with 200 μ l of ¹²⁵I-labeled tracer. Radioac-

tivity was counted with a gamma counter (Clini Gamma 1272; Wallac Oy, Turku, Finland). IFN- γ activity is expressed as NIH units. The detection limit of the test is 18 U/l. IFN- γ concentrations of the patients were compared to healthy HIV-1 seronegative controls [8, 11].

Serum neopterin concentrations were measured using radioimmunoassay (RIAid, Henning-Berlin, Berlin, F.R.G.). Serum (50 μ l) was incubated with 100 μ l neopterin antiserum for 1 h at room temperature. Then 100 μ l of ¹²⁵I-labeled tracer was added, followed by incubation for 1 h. Aqueous polyethylene glycol 6000 solution (2 ml; 60 g/l) was added. After centrifugation at 2000 \times g for 10 min, radioactivity was counted using the gamma counter. The detection limit was 1 nmol/l. Neopterin concentrations of the patients were compared to those of healthy HIV-1 seronegative controls [12].

Tryptophan and kynurenine concentrations were measured by reverse-phase high pressure liquid chromatography as described using on-line deproteinization of samples without precipitation [13]. Tryptophan was measured by fluorescence detection at 285 nm excitation wavelength and 365 nm emission wavelength; kynurenine was measured by UV-absorption of 360 nm wavelength [14]. Tryptophan and kynurenine concentrations of patients were compared to healthy HIV-seronegative controls [14].

Statistical evaluation of grouped data was done employing the Wilcoxon rank test. Correlation analysis was done using Spearman's rank correlation coefficients. The results were confirmed by computing linear regression coefficients (data not shown).

TABLE 1

Serum IFN- γ , neopterin, tryptophan and kynurenine concentrations in 42 HIV-1 seropositive patients compared to healthy HIV-1 seronegative controls [11, 10, 12]

	HIV-1 seropositive patients			HIV-1 seronegative controls
	mean \pm S.E.	median	15th–75th percentile	mean \pm S.E. (n)
IFN- γ (U/l)	259 \pm 70*	102	57–233	23.5 \pm 1.7 (76)
Neopterin (nmol/l)	24.0 \pm 2.8*	19.1	14.9–28.8	5.34 \pm 0.14 (359)
Tryptophan (μ mol/l)	57.0 \pm 2.3*	57.0	41.6–72.0	91.0 \pm 6.63 (11)
Kynurenine (μ mol/l)	3.45 \pm 0.14*	3.20	2.80–3.92	2.31 \pm 0.23 (11)

*Significantly different from healthy HIV-1 seronegative controls ($P < 0.01$; Wilcoxon rank test).

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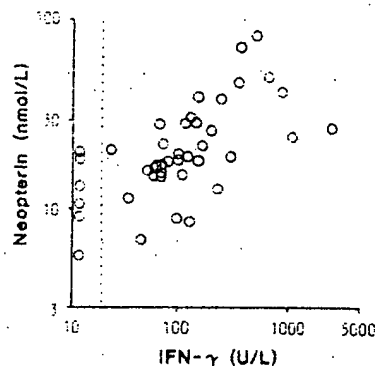


Fig. 1. Serum IFN- γ and neopterin concentrations (Spearman's rank correlation coefficient $r_s = 0.583$, $P < 0.001$). Dotted line shows the lower limit of detection for IFN- $\gamma = 18$ U/L.

4. Results

Thirty-six of 42 (85.7%) HIV-1-seropositives had detectable IFN- γ in serum. The IFN- γ concentrations were significantly higher compared to HIV-1 seronegative controls (Table 1); 23 (54.8%) had increased concentrations compared to the upper normal limit (95th percentile of HIV-1-seronegative blood donors = 100 U/L) [8, 11]. Patients also had higher neopterin and kynurenine concentrations in serum compared to healthy HIV-1 seronegative controls (Table 1). In contrast, tryptophan levels were lower in HIV-1 seropositives.

Within our patient group, serum neopterin and IFN- γ levels were higher in WR5/6 patients (mean \pm S.E.M.; neopterin, 27.5 ± 3.05 nmol/L; IFN- γ , 312 ± 100 U/L) than in WR2-4 patients (neopterin, 18.7 ± 4.30 , $p = 0.011$; IFN- γ , 178 ± 76.8 , $p = 0.092$). Kynurenine was nearly identical in both

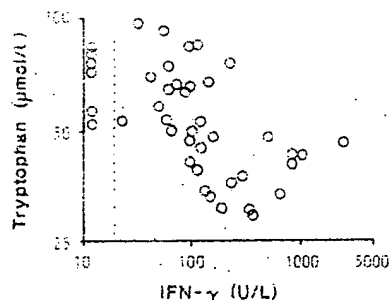


Fig. 2. Correlation between serum IFN- γ and tryptophan concentrations in 42 HIV-1 seropositive individuals (Spearman's rank correlation coefficient $r_s = -0.0616$, $P < 0.001$). Dotted line shows the lower limit of detection for IFN- $\gamma = 18$ U/L.

TABLE 2

Associations (Spearman rank correlation coefficients and P values) between serum IFN- γ , tryptophan, kynurenine and neopterin concentrations and kynurenine:tryptophan ratios in 42 HIV-1 seropositive patients.

	Tryptophan	Kynurenine	Neopterin
IFN- γ	$r = -0.616$ $P < 0.001$	$r = 0.114$ $P > 0.1$	$r = 0.583$ $P < 0.001$
Tryptophan		$r = 0.040$ $P > 0.1$	$r = 0.716$ $P < 0.001$
Kynurenine			$r = 0.411$ $P = 0.008$

groups (WR2-4, 3.4 ± 0.29 μ mol/L; WR5,6, 3.5 ± 0.16). Serum tryptophan was slightly lower in WR5,6 patients (54.1 ± 3.0 μ mol/L) than in WR2-4 patients, but the difference was not significant (61.5 ± 6.03 ; $P = 0.15$).

Several significant correlations were seen between the variables studied (Table 2). Serum IFN- γ concentrations correlated positively with serum neopterin levels (Fig. 1) and negatively to serum tryptophan levels (Fig. 2). No correlation existed between IFN- γ and kynurenine concentrations (Table 2) whereas a significant correlation was found between serum IFN- γ and the kynurenine:tryptophan ratio ($r = 0.615$, $P < 0.001$). Serum neopterin levels correlated positively to kynurenine concentrations (Fig. 3) and to the kynurenine:tryptophan ratio ($r = 0.842$, $P < 0.001$).

No difference was observed with respect to IFN- γ , neopterin, tryptophan, and kynurenine levels in pa-

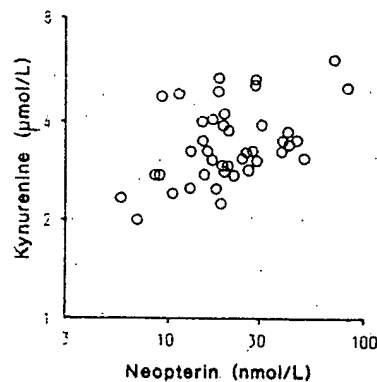


Fig. 3. Correlation between serum neopterin and kynurenine concentrations (Spearman's rank correlation coefficient $r_s = 0.411$, $P < 0.008$).

tients either treated or not treated with AZT. However, during follow-up of patients when treatment with AZT was initiated, we observed decreasing IFN- γ , neopterin and kynurenine concentrations; in contrast, tryptophan levels increased (data not shown).

5. Discussion

Reduced capacity of T cells to respond to antigenic and mitogenic stimulation *in vitro* is frequent in patients with HIV-1 infection. Nearly all patients with advanced stages of infection present with severe immune deterioration [1]. T cells of these patients show almost diminished or absent production of cytokines such as IL-2 and IFN- γ upon stimulation [1, 15]. This *in vitro* finding agrees with partial or complete skin test anergy of patients. In contrast, circulating IFN- γ concentrations are increased in approximately half of our patients with HIV-1 infection compared to healthy HIV-1 seronegative controls. This result confirms earlier data [8]. Also Murray et al. found similar concentration ranges of IFN- γ in HIV-1 seropositives using radioimmunoassay [15].

Our data further show that IFN- γ is biologically active in HIV-1 seropositives. Significantly decreased tryptophan levels found simultaneously with increased kynurenine levels in our patients indicate active degradation of tryptophan via induction of IDO. The expression of IDO as well as activation of the enzyme can be induced by IFN- γ [10]. In addition, IFN- γ correlates positively to neopterin concentrations. Increased neopterin levels reflect induction of GTP-cyclohydrolase I (EC 3.5.4.16), which is strongly inducible by IFN- γ *in vitro* [12].

The decrease of tryptophan concentrations is much stronger than the increase of kynurenine in our study, and the correlations between IFN- γ and neopterin are much stronger with tryptophan than with kynurenine. This can be explained by the fact that kynurenine once produced is rapidly metabolized. The tryptophan metabolite quinolinic acid was found to be increased during the course of HIV-1 infection [16]. In agreement with this, IDO is induced by IFN- γ ; the subsequent enzymes downstream the tryptophan degradation pathway are constitutive in various human cells [17].

Changes of tryptophan concentrations were

found to correlate to neurological/psychiatric symptoms in patients with HIV-1 infection [14]. Enhanced degradation of the essential amino acid tryptophan may be the reason for this observation: (1) Reduced formation of the neurotransmitter 5-hydroxytryptamine [18], and (2) increased formation of neurotoxic tryptophan metabolites such as quinolinic acid may contribute to precipitation of symptoms in HIV-1 seropositives [16].

The finding of circulating IFN- γ in HIV-1 seropositives could be also relevant with respect to the pathogenesis of AIDS. The presence of IFN- γ indicates that T cells of patients with HIV-1 infection are chronically stimulated. Infection with HIV-1 itself or by secondary pathogens may be responsible for immune stimulation which in turn may induce and support replication of HIV-1 in patients [5, 19]. Our data demonstrate that (1) IFN- γ is increased in HIV-1 infection and (2) metabolic changes can be detected which can be referred to the activity of IFN- γ . There exists an obvious discrepancy to *in vitro* findings on IFN- γ . However, this observation is not unique to HIV-1 infection. In several other diseases which are associated with chronic immune activation such as graft versus host disease, autoimmune disorders and other chronic infections, reduced *in vitro* responsiveness of peripheral blood mononuclear cells is found [20].

Acknowledgements

This work was financially supported by the Austrian Research Funds 'Zur Förderung der wissenschaftlichen Forschung', p 7910.

References

- [1] Fauci, A. S. (1988) *Science* 239, 617.
- [2] Gupta, S. and Licorish, K. (1984) *N. Engl. J. Med.* 310, 1530.
- [3] Eyster, M. E., Goedert, J. J., Poon, M. C. and Preble, O. T. (1983) *N. Engl. J. Med.* 309, 583.
- [4] Prince, H. E., Kleinmann, S. and Williams, A. E. (1988) *J. Immunol.* 140, 1139.
- [5] Fuchs, D., Hausen, A., Reibnegger, G., Werner, E. R., Dierich, M. P. and Wachter, H. (1988) *Immunol. Today* 9, 150.
- [6] Fahey, J. L., Taylor, J. M. G., Detels, R., Hofmann, B., Nishanian, P. and Giorgi, J. V. (1990) *N. Engl. J. Med.* 322, 166.
- [7] Fuchs, D., Jäger, H., Popescu, M., Reibnegger, G., Werner, E. R., Dierich, M. P., Kaboth, W., Titz, G. P. and Wachter, H.

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- H. (1990) Immunol. Lett. 26, 75.
- [8] Fuchs, D., Hausen, A., Reibnegger, G., Werner, E. R., Werner-Felmayer, G., Dierich, M. P. and Wachter, H. (1989) J. AIDS 2, 158.
- [9] Werner, E. R., Fuchs, D., Hausen, A., Jäger, H., Reibnegger, G., Werner-Felmayer, G., Dierich, M. P. and Wachter, H. (1988) Biol. Chem. Hoppe-Seyler 369, 337.
- [10] Yoshida, R., Imanishi, J., Toku, J. and Hayaishi, O. (1981) Proc. Natl. Acad. Sci. USA, 78, 129.
- [11] Woloszczuk, W. (1985) Clin. Chem. 31, 1090.
- [12] Wachter, H., Fuchs, D., Hausen, A., Reibnegger, G. and Werner, E. R. (1989) Adv. Clin. Chem. 27, 81.
- [13] Werner, E. R., Fuchs, D., Hausen, A., Reibnegger, G. and Wachter, H. (1987) Clin. Chem. 33, 2028.
- [14] Fuchs, D., Möller, A. A., Reibnegger, G., Stöckle, E., Werner, E. R. and Wachter, H. (1990) J. AIDS 3, 873.
- [15] Murray, H. W., Scavuzzo, D., Jacobs, J. L., Kaplan, M. H., Libby, D. M., Schindler, J. and Roberts, R. B. (1987) J. Immunol. 138, 2457.
- [16] Heyes, M. P., Rubinow, D., Lane, C. and Markey, S. P. (1989) Ann. Neurol. 26, 275.
- [17] Werner-Felmayer, G., Werner, E. R., Fuchs, D., Hausen, A., Reibnegger, G. and Wachter, H. (1989) Biochim. Biophys. Acta 1012, 140.
- [18] Launay, J. M., Copel, L., Callebert, J., Corvaia, N., Lepage, E., Bricaire, F., Saal, F. and Peries, J. (1988) J. AIDS 1, 324.
- [19] Zagury, D., Bernard, J., Leonard, R., Cheynier, R., Feldman, M., Sarin, P. S. and Gallo, R. C. (1986) Science 231, 850.
- [20] Fuchs, D., Malkowsky, M., Reibnegger, G., Werner, E. R., Forni, G. and Wachter, H. (1989) Immunol. Lett. 23, 103.

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Journal of Neuroimmunology, 40 (1992) 71-80
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Inter-relationships between quinolinic acid, neuroactive kynurenines, neopterin and β_2 -microglobulin in cerebrospinal fluid and serum of HIV-1-infected patients

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(Received 22 January 1992)

(Revised, received 26 March 1992)

(Accepted 27 March 1992)

Key words: Quinolinic acid; Kynurenic acid; L-Tryptophan; L-Kynurenine; Indoleamine-2,3-dioxygenase; Pterine; β_2 -Microglobulin; NMDA receptor; AIDS dementia complex; Interferon- γ

Summary

Quinolinic acid (QUIN) is an neurotoxic *N*-methyl-D-aspartate receptor agonist and an L-tryptophan metabolite of the kynurenine pathway. Increased concentrations of QUIN occur in both cerebrospinal fluid (CSF) and blood of patients infected with human immunodeficiency virus (HIV)-1, particularly those with neurologic disturbances. In the present study of HIV-1 infected patients in Walter Reed stages 4, 5 and 6, reductions in L-tryptophan accompanied proportional increases in L-kynurenine and QUIN in both serum and CSF. Further, close inter-correlations exist between QUIN, kynurenic acid and L-kynurenine with both β_2 -microglobulin and neopterin in CSF and serum. These correlations support the hypotheses that the kynurenine pathway is activated in association with inflammation and induction of indoleamine-2,3-dioxygenase. There were no relationships between CSF QUIN, L-kynurenine or kynurenic acid with the ratio of serum:CSF albumin concentrations, which indicates that the increases in CSF QUIN, L-kynurenine or kynurenic acid were not dependent on a breakdown of the blood-brain barrier. Kynurenic acid is also a kynurenine pathway metabolite that can attenuate the excitotoxic effects of QUIN when present in higher molar concentrations. While CSF kynurenic acid levels were increased in HIV-1-infected patients, the magnitude of the increases were smaller than those of QUIN and the molar concentrations of kynurenic acid were consistently lower than QUIN by at least one order of magnitude. We conclude that immune activation increases the levels of neuroactive kynurenines within the central nervous system of HIV-1-infected patients secondary to activation of indoleamine-2,3-dioxygenase.

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EXHIBIT B

①

Introduction

Substantially increased concentrations of the excitotoxin and kynurenine pathway metabolite, quinolinic acid (QUIN), are found in the cerebrospinal fluid (CSF) of patients infected with the human immunodeficiency virus (HIV-1; Heyes et al., 1989), particularly among patients with the AIDS dementia complex, aseptic meningitis or opportunistic central nervous system conditions (Heyes et al., 1991a). QUIN is an agonist of *N*-methyl-D-aspartate receptors and an excitotoxin. Notably, the concentrations achieved in the CSF of HIV-1-infected patients (Heyes et al., 1991a) exceeded levels reported to be neurotoxic in vitro (Giulian et al., 1990; Whetsell and Schwarcz, 1989). Consequently, we have postulated that QUIN may be involved in the neurologic complications of HIV-1 infection, including

the AIDS dementia complex (Heyes et al., 1989, 1991a). The potential role of *N*-methyl-D-aspartate receptors in mediating neuronal damage in HIV-1-infection has been further highlighted by subsequent in vitro studies (Giulian et al., 1990; Lipton et al., 1991).

Other factors, however, may influence the neurologic effects of QUIN and other *N*-methyl-D-aspartate receptor agonists. In particular, the related kynurenine pathway metabolite kynurenic acid (KYNA) can attenuate the excitotoxic effects of QUIN by virtue of its antagonist effects on excitatory amino acid receptors, including *N*-methyl-D-aspartate receptors (Foster et al., 1984). Therefore, the balance between the concentrations of QUIN and KYNA may influence whether the excitotoxic effects of QUIN or other neurotoxins are manifest. The present study sought to determine whether the levels of KYNA are in-

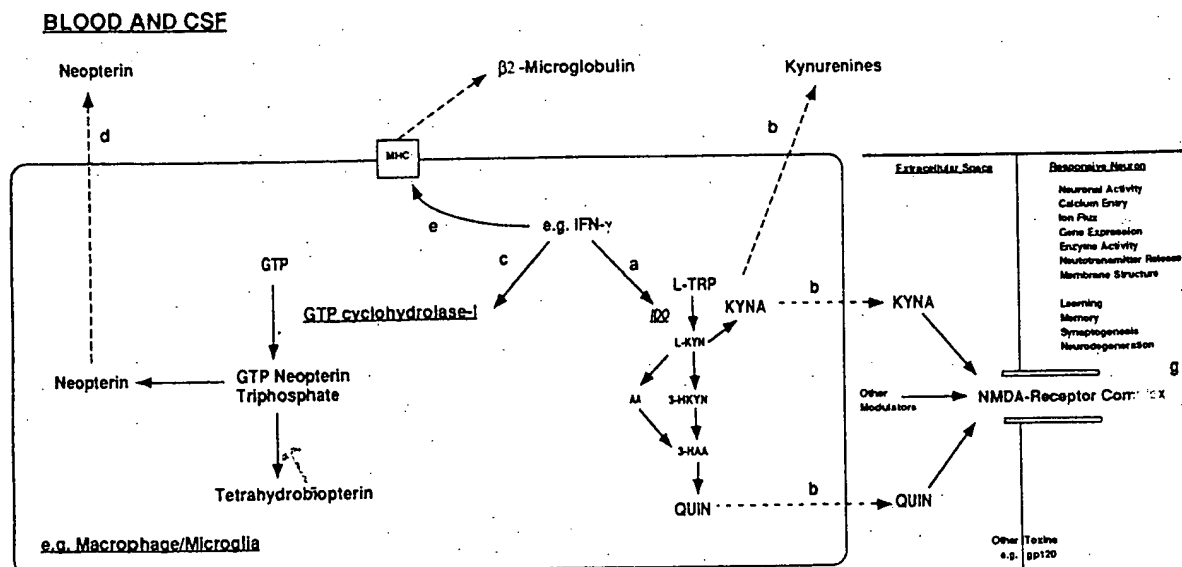


Fig. 1. Model of the metabolic relationships between selected cytokines, kynurenine pathway metabolites, neopterin and β_2 -microglobulin and the potential role of *N*-methyl-D-aspartate (NMDA) in neuronal dysfunction and neurodegeneration in HIV-1 infection and other inflammatory diseases. Broken lines represent transfer of substrate from one metabolic compartment to another, solid lines represents metabolic reactions or effects of agents on enzymes or receptors. Enzymes are underlined. Interferon- γ (IFN- γ) and other cytokines increase indoleamine-2,3-dioxygenase (IDO) activity in macrophages and other cells (a) and increase the conversion of L-tryptophan to L-kynurenine (L-KYN), KYNA, anthranilic acid (AA), 3-hydroxykynurenine (3-HKYN), 3-hydroxyanthranilic acid (3-HAA) and QUIN which may enter the blood, CSF and extracellular fluid space of the brain (b). IFN- γ also activates guanosine triphosphate (GTP) cyclohydrolase-I and increases the synthesis of neopterin (c) which also appears in the blood and CSF (d). The release of β_2 -microglobulin is also increased by IFN- γ (e). Increased concentrations of QUIN, kynurenic acid and other modulators of *N*-methyl-D-aspartate receptor activity may induce neuronal dysfunction and nerve cell death and thereby result in neurologic symptoms (g). Strategies to alter the synthesis of *N*-methyl-D-aspartate receptor ligands or attenuate their effects offer new approaches to therapy in inflammatory diseases.

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creased in the CSF, and investigate the relationship of KYNA to QUIN and other neuroactive kynurenines in the CSF of HIV-1-infected patients.

The increases in CSF and serum QUIN in HIV-1-infected patients have been attributed to induction of indoleamine-2,3-dioxygenase, the first enzyme of the kynurenine pathway which converts L-tryptophan to L-kynurenine (Fig. 1). The increases in both brain and lung indoleamine-2,3-dioxygenase activity in non-human primate models of AIDS are consistent with this hypothesis (Saito et al., 1991a). To further investigate the role of indoleamine-2,3-dioxygenase in changing kynurenine pathway metabolism, we used the concentrations of L-tryptophan and L-kynurenine in blood and CSF as an index of indoleamine-2,3-dioxygenase activity (Fuchs et al., 1990; Heyes and Lackner, 1990; Saito et al., 1991a) and determined their relationships to QUIN and KYNA. In addition, because host immune mediators such as interferon- γ and tumor necrosis factor- α may increase indoleamine-2,3-dioxygenase activity, QUIN production, neopterin synthesis, and β_2 -microglobulin expression (Pfefferkorn and Guyre, 1984; Byrne et al., 1986; Bianchi et al., 1988; Saito et al., 1991b; Heyes et al., 1991, 1992a, b), we examined the relationship of kynurenine pathway metabolites with neopterin and β_2 -microglobulin, which are 'markers' of immune stimulation (see Fig. 1). The potential role of disruption of the blood-brain barrier was studied by measuring the CSF:serum albumin ratio.

Materials and methods

Subjects studied

Samples of both CSF and serum were collected from HIV-1-infected patients who were being studied at the Memorial Sloan-Kettering Cancer Center. Blood was collected from an arm vein and serum was isolated by centrifugation. CSF was collected from the lumbar sac. The clinical characteristics of these patients have been described previously (Brew et al., 1989, 1990; Heyes et al., 1991a). The systemic disease state of these subjects was classified according to the

Walter Reed (WR) Staging system (WR 4-5, $n = 40$; WR 6, $n = 39$ (Redfield et al., 1986). AIDS dementia complex scores (0-4) were determined according to published criteria (Brew et al., 1989). The number of patients in each group were: demented, WR 4-5, $n = 20$; WR 6, $n = 28$; or not demented, WR 4-5, $n = 20$; WR 6, $n = 11$. Patients were studied in various stages of systemic and central nervous system disease. None of the patients had clinical aseptic meningitis (Hollander and Stringari, 1987), demonstrable opportunistic central nervous system infections or neoplasms. Because the samples were obtained before the approval or widespread use of zidovudine (azidothymidine or AZT), none of the patients were receiving anti-retroviral therapies at the time of sample collection. Control subjects were 22 age-matched healthy and neurologically unimpaired volunteers.

Biochemical measurements

Samples were assayed by experienced laboratory personnel, using established and verified methods, without prior knowledge to the patients' viral or clinical status. QUIN was quantified by electron capture negative chemical ionization gas chromatography/mass spectrometry which uses [^{18}O]QUIN as internal standard, rather than structural isomers or chemical analogs (Heyes and Markey, 1988). The concentrations of KYNA, L-kynurenine and L-tryptophan in CSF and serum were quantified by high performance liquid chromatography with either fluorescence detection (Heyes and Quearry, 1990) or ultraviolet light absorbance spectrometry (adapted from Holmes, 1988) or electrochemical detection (Heyes and Markey, 1988) respectively. Generally, measures were made within the same assay run. However, where more than one assay run was done, selected samples from previous assays were included in subsequent procedures to ensure replicate values were within established and acceptable variability limits. In no case could group mean differences be attributed to systematic assay errors. In other studies, no gradients for QUIN, KYNA or L-kynurenine have been noted along the CSF axis (Mouradian et al., 1989; Heyes and Sunderland, unpublished observations). β_2 -Microglobulin and neopterin were mea-

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sured in CSF and serum by radioimmunoassay (Electronuclonics-Diagnostics, Piscataway NJ, and Henning, Berlin, respectively). The integrity of the blood-brain barrier was assessed by measuring the ratio of CSF : serum albumin concentrations (Brew et al., 1989). Measures of albumin, IgG and white blood cell counts were done by routine laboratory methods.

Statistical analyses

Results were analysed by one-way analysis of variance with Dunnett's *t*-test for multiple comparisons (Feldman et al., 1987). All regression analyses were done using the method of least squares after logarithmic transformation (Table 1). Non-parametric correlation coefficients were calculated as the Spearman Rank Correlation coefficient. Values presented are mean \pm one standard error of the mean or percent of control subjects unless otherwise stated.

Results

Relationships between L-kynurenine, L-tryptophan, KYNA and QUIN

The concentrations of CSF L-tryptophan, L-kynurenine, KYNA and QUIN concentrations as well as the ratio of QUIN:KYNA in HIV-1-infected patients are presented in Fig. 2. Systemic disease was classified as WR 4-5 or WR 6 (AIDS) and neurologic disease was classified as either not demented (AIDS dementia complex scores ≤ 0.5) or demented (AIDS dementia complex scores ≥ 1). Values are expressed as a percent of age-matched control subjects and plotted on a logarithmic scale. The increases in L-kynurenine, KYNA and QUIN were largest in both groups of demented patients compared to same stage non-demented patients. The increases in CSF QUIN in demented patients was approximately the same in the WR 4-5 patients as in the WR 6 patients. However, the highest ratio of QUIN:KYNA in the CSF was found in the demented WR 6 patients. In the HIV-1-infected patients taken collectively, there were significant inter-correlations between the concentrations of L-kynurenine, KYNA, QUIN, neopterin, β_2 -microglobulin and IgG in the CSF (Table 1). In contrast, among the

TABLE 1

CORRELATION COEFFICIENTS BETWEEN QUIN, KYNA, L-KYN, L-TRP, NEOPTERIN AND β_2 -MICROGLOBULIN IN HIV-1-INFECTED PATIENTS

	Correlation coefficient (r)	P-value
CSF		
log QUIN vs. log KYNA	+0.86	$P < 0.0001$
log QUIN vs. log L-kynurenine	+0.76	$P < 0.0001$
log QUIN vs. L-tryptophan	-0.40	$P < 0.005$
log QUIN vs. log neopterin	+0.71	$P < 0.0001$
log QUIN vs. log β_2 -microglobulin	+0.69	$P < 0.001$
log QUIN vs. log IgG	+0.46	$P < 0.005$
log KYNA vs. log L-kynurenine	+0.85	$P < 0.0001$
log KYNA vs. L-tryptophan	-0.50	$P < 0.01$
log KYNA vs. log neopterin	+0.79	$P < 0.0001$
log KYNA vs. log β_2 -microglobulin	+0.74	$P < 0.0001$
log KYNA vs. log IgG	+0.80	$P < 0.002$
log L-kynurenine vs. L-tryptophan	-0.41	$P < 0.02$
log L-kynurenine vs. log neopterin	+0.56	$P < 0.005$
log L-kynurenine vs. log β_2 -microglobulin	+0.58	$P < 0.0001$
log L-kynurenine vs. log IgG	0.34	$P < 0.05$
Serum		
log QUIN vs. log L-kynurenine	+0.75	$P < 0.0001$
log QUIN vs. L-tryptophan	-0.31	$P < 0.02$
log QUIN vs. log neopterin	+0.70	$P < 0.0001$
log QUIN vs. log β_2 -microglobulin	+0.68	$P < 0.0001$
L-KYN vs. L-tryptophan	-0.32	$P < 0.01$
L-KYN vs. log neopterin	+0.69	$P < 0.0001$
L-KYN vs. log β_2 -microglobulin	+0.54	$P < 0.0002$
L-Tryptophan vs. log neopterin	-0.43	$P < 0.001$
L-Tryptophan vs. log β_2 -microglobulin	-0.36	$P < 0.02$

WR 4-6 patients, there was an inverse correlation between CSF L-tryptophan with CSF QUIN, KYNA and L-kynurenine (Table 1).

There were no significant differences in serum QUIN, L-kynurenine or L-tryptophan between the four sub-groups of HIV-1-infected patients and the data were pooled for comparison to control subjects. The percent changes in the serum parameters were substantially less than the percent changes in the CSF. Serum L-kynurenine concentrations were increased in the WR 4-6 patients ($4.14 \pm 0.25 \mu\text{M}$, $n = 44$) compared to controls

($2.19 \pm 0.19 \mu\text{M}$) increased substrate pathway. There were no significant differences between L-tryptophan and QUIN while there was a significant difference between L-kynurenine (Table 1). Serum L-kynurenine in the WR 4-6

10000

1000

% of Control Subjects

10

Fig. 2. CSF L-tryptophan, L-kynurenine, KYNA and QUIN concentrations in HIV-1-infected patients with and without dementia (WR 4-5 and WR 6) compared to age-matched normal or red

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 $P < 0.0005$

$P < 0.0001$
 $P < 0.01$
 $P < 0.001$
 $P < 0.0001$
 $P < 0.002$

$P < 0.02$
 $P < 0.005$

$P < 0.0001$
 $P < 0.005$

$P < 0.0001$
 $P < 0.02$
 $P < 0.0001$
 $P < 0.0001$

$P < 0.01$
 $P < 0.0001$
 $P < 0.0002$

$P < 0.001$

$P < 0.002$

($2.19 \pm 0.19 \mu\text{M}$, $P < 0.0001$), consistent with increased substrate flux through the kynurenine pathway. There was an inverse correlation between L-tryptophan and L-kynurenine concentrations and QUIN levels in the serum (Table 1), while there was a direct correlation in serum between L-kynurenine with QUIN concentrations (Table 1). Serum L-tryptophan levels were lower in the WR 4-6 patients compared to the controls

($40.2 \pm 1.5 \mu\text{M}$, $n = 107$ vs. $70.9 \pm 6.9 \mu\text{M}$, $P < 0.0001$). Although serum L-tryptophan levels influence brain L-tryptophan uptake (Fernstrom, 1983), there was no significant correlation between the concentrations of L-tryptophan in CSF and serum in the WR 4-6 patients ($r = 0.21$, $P = 0.12$). This may indicate that the central and systemic compartments are being influenced independently. There was a significant correlation

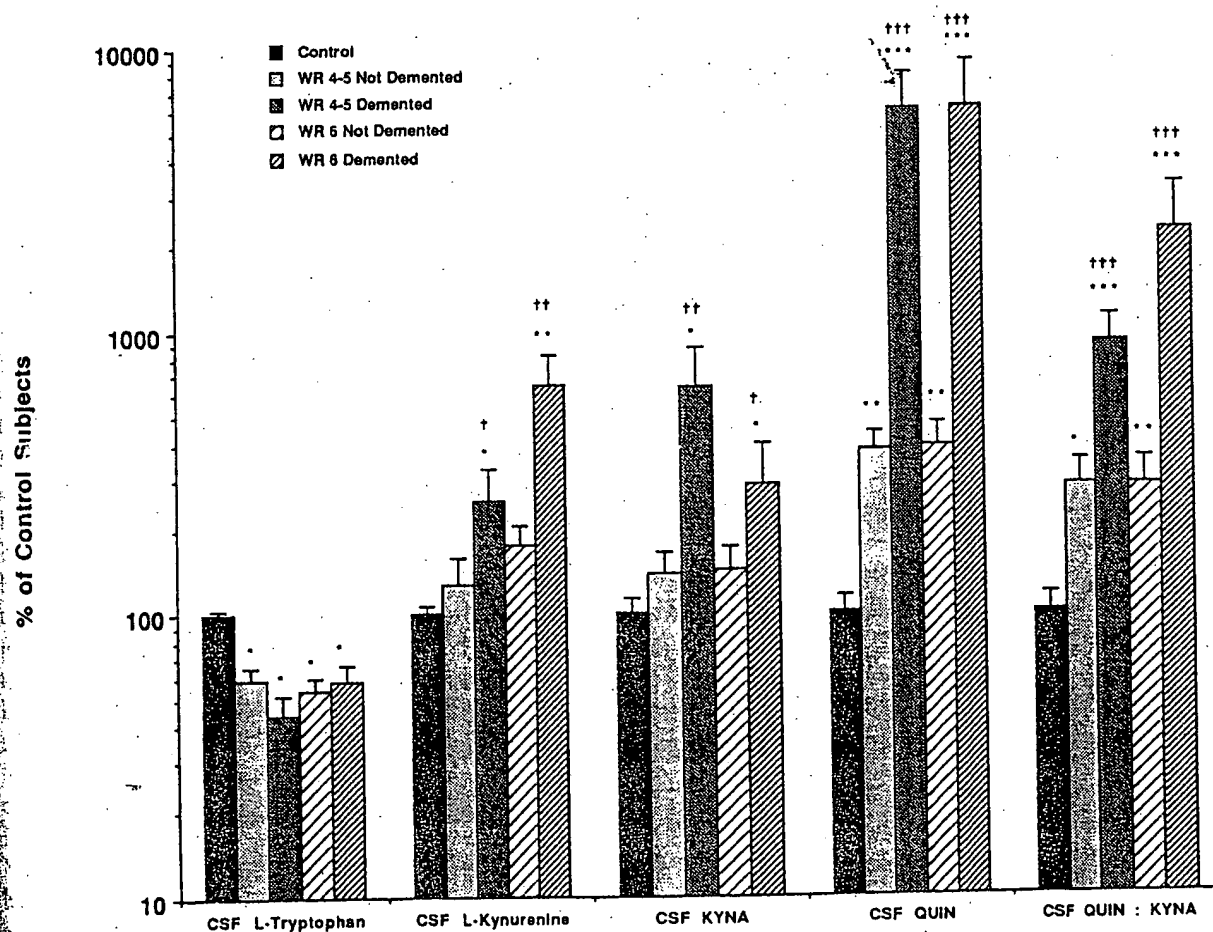


Fig. 2. CSF L-tryptophan, L-kynurenine, KYNA and QUIN concentrations in untreated HIV-1-infected patients who were either demented (WR 4-5, $n = 20$; WR 6, $n = 28$) or not demented (WR 4-5, $n = 20$; WR 6, $n = 11$). No patients had opportunistic CNS conditions, aseptic meningitis or were being treated with anti-retroviral drugs. Values presented are expressed as a percent of age-matched neurologically normal volunteers (L-tryptophan, $2.32 \pm 0.09 \mu\text{M}$; L-kynurenine, $52.9 \pm 3.1 \text{ nM}$; KYNA, $3.49 \pm 0.44 \text{ nM}$ and QUIN $22.0 \pm 3.3 \text{ nM}$). * $P < 0.05$, ** $P < 0.001$, *** $P < 0.005$, **** $P < 0.0001$ versus control; † $P < 0.05$, †† $P < 0.005$, ††† $P < 0.0001$ versus respective not demented WR stage. Note: there are no gender differences, and no gradients for QUIN, KYNA, L-kynurenine or L-tryptophan along the CSF axis. Furthermore, brain atrophy, neurodegeneration, dementia or motor disturbances cannot account for these increases as CSF L-tryptophan, L-kynurenine, KYNA and QUIN concentrations are either normal or reduced in patients with either Huntington's disease, Alzheimer's disease, complex partial seizures, bipolar and unipolar depression, bulimia nervosa, anorexia nervosa or schizophrenia (Heyes et al., Brain, in press).

inverse correlation
with CSF QUIN,

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was reduced to controls

between CSF and serum L-kynurenine concentrations ($r = 0.65$, $P < 0.0001$).

Relationships of kynurenine pathway metabolites to AIDS dementia complex scores

As well as the significant correlations between CSF QUIN, neopterin and β_2 -microglobulin with AIDS dementia complex scores noted previously (Brew et al., 1989, 1990; Heyes et al., 1991a), significant correlations between CSF L-kynurenine ($P < 0.002$) and the CSF QUIN:KYNA ratio ($P < 0.05$) with AIDS dementia complex scores were also noted.

Relationships of kynurenine pathway metabolites to neopterin, β_2 -microglobulin and blood-brain barrier integrity

The inter-relationships between QUIN, KYNA and L-kynurenine with both neopterin, β_2 -microglobulin and IgG are summarized in Table 1, and support a link between kynurenine pathway metabolism and immune stimulation within both the brain and periphery (Fig. 1). In CSF and serum, both neopterin and β_2 -microglobulin concentrations were higher in the WR 4-6 patients compared to controls (see Brew et al., 1989, 1990). In the WR 4-6 patients, there were significant correlations between concentrations of neopterin, β_2 -microglobulin and concentrations of CSF IgG with QUIN, KYNA and L-kynurenine.

There were no significant differences in the CSF:serum albumin ratio in the four groups of HIV-1-infected patients. There were also no significant correlations between CSF:serum albumin ratio with CSF QUIN, KYNA, L-kynurenine or L-tryptophan, except the modest correlation with CSF QUIN noted only in demented patients taken collectively (see Heyes et al., 1991a). CSF white blood cell counts were < 10 cells/ml in 93% of samples studied and there were no correlations between CSF QUIN, KYNA, L-kynurenine or L-tryptophan with CSF white blood cell counts.

Serum QUIN and L-kynurenine levels were significantly correlated with both serum β_2 -microglobulin and neopterin in the WR 4-6 patients. Conversely, serum L-tryptophan levels were inversely correlated with serum QUIN, L-kynurenine, β_2 -microglobulin and neopterin concentrations.

Discussion

QUIN and other putative activators of N-methyl-D-aspartate receptors have been implicated in the etiology of HIV-1 neurologic disease (Heyes et al., 1988, 1989, 1991a; Giulian et al., 1990; Lipton et al., 1991). Importantly, QUIN has also been proposed as a neurotoxin in other inflammatory neurologic diseases, because of the sensitivity of indoleamine-2,3-dioxygenase to activation by endotoxin and interferon- γ , and because substantial increases in CSF QUIN concentrations are found in patients with inflammatory neurologic disease (Takikawa et al., 1986, 1988; Heyes et al., 1988; Heyes and Lackner, 1990; Saito et al., 1991b; Halperin and Heyes, 1992). The purpose of the present study was to determine whether the CSF levels of KYNA, a modulator of QUIN neurotoxicity, are also increased in HIV-1-infected patients. We also investigated potential mechanisms that may be involved in increasing QUIN synthesis. The results demonstrate that the substantial increases in CSF QUIN levels in HIV-1-infected patients are accompanied by parallel increases in KYNA. The results strongly support activation of indoleamine-2,3-dioxygenase in direct proportion to the degree of intrathecal immune activation.

Studies in experimental animals have shown that KYNA can protect neurons against the excitotoxic effects of QUIN. However, a ratio of up to 3:1 in favor of KYNA is needed for maximal protection (Boegman et al., 1990; Foster et al., 1984). While it is not known whether this relationship between QUIN, KYNA and excitotoxicity applies to humans, it is of note that the ratio of QUIN:KYNA actually favors QUIN in normal subjects (8.71) and is further increased in the HIV-1 infected patients (Fig. 2). Analogous increases in the ratio of QUIN:KYNA have also been noted in primate models of AIDS and septicemia (Heyes et al., 1990a, b, 1992; Heyes and Lackner, 1990). Therefore, the ratio of QUIN:KYNA favours QUIN excitotoxicity. Also, while increases in brain KYNA levels may be viewed as potentially beneficial in attenuating the excitotoxic effects of QUIN and other excitotoxins, it is possible that KYNA as an antagonist of excitatory amino acid neurotransmitters may con-

tribute to the neurotatory amino acid vation. QUIN may neurotransmission logic deficits by a

A model of pos in CSF and serum well as other cor also applies to th L-kynurenine (Fig et al., 1990a, 199 KYNA may be c had been taken i blood (Gal and Quearry, 1990). (Heyes and Que tion of indolea creases in the oxygenase in bo infected with the or the type-D re systemic and cen Saito et al., 199 the present study QUIN and L-kyr fected patients. subjects. is in : indoleamine-2,3. Further, the pc levels of L-KYN creased substra pathway within that the magni kynurenine and than the incre: has been note conditions (Hal Lackner, 1990; Nevertheless, s be important and QUIN, pa immune activa septicemia (He

The model are a principle 1991a). Infiltr microglia are feature of HIV many other

tribute to the neurologic deficits by blocking excitatory amino acid receptors during immune activation. QUIN may also interfere with excitatory neurotransmission and thereby produce neurologic deficits by a non-cytolytic mechanism.

A model of possible mechanisms for increases in CSF and serum QUIN in HIV-1 infection, as well as other conditions of immune activation, also applies to the increases in CSF KYNA and L-kynurenine (Fig. 1; Takikawa et al., 1986; Heyes et al., 1990a, 1991a; Heyes and Lackner, 1990). KYNA may be derived from L-kynurenine that had been taken up either by the brain from the blood (Gal and Sherman, 1980; Heyes and Quearry, 1990), or synthesized within the brain (Heyes and Quearry, 1990) secondary to activation of indoleamine-2,3-dioxygenase. The increases in the activity of indoleamine-2,3-dioxygenase in both lung and brain of macaques infected with the Simian immunodeficiency virus or the type-D retrovirus are consistent with both systemic and central synthesis (Heyes et al., 1990b; Saito et al., 1991a). The inverse correlations in the present study between L-tryptophan with both QUIN and L-kynurenine in the CSF of HIV-1-infected patients, but not in the CSF of control subjects, is in accordance with increased brain indoleamine-2,3-dioxygenase activity (Table 1). Further, the positive correlations between CSF levels of L-KYN, KYNA and QUIN support increased substrate flux through the kynurenine pathway within the CNS (Table 1). It is of note that the magnitude of the increases in CSF L-kynurenine and QUIN are substantially greater than the increases in serum. This phenomenon has been noted in other inflammatory disease conditions (Halperin and Heyes, 1992; Heyes and Lackner, 1990; Heyes et al., 1990a, b, 1992a). Nevertheless, substrates derived from blood may be important sources of L-kynurenine, KYNA and QUIN, particularly if the levels of systemic immune activation is marked, for example during septicemia (Heyes and Lackner, 1990).

have shown against the excitotoxicity, a ratio of up to 10 for maximal excitotoxicity. Foster et al., 1990, whether this relationship is excitotoxic or that the ratio of QUIN in CSF to L-tryptophan is increased in the CSF of AIDS and septicemia. Analogous increases in QUIN have also been found in AIDS and septicemia (Heyes and Lackner, 1990; Heyes et al., 1990a, b, 1992a). The ratio of QUIN to L-tryptophan in CSF is also increased in the CSF of patients with AIDS and septicemia. Also, levels of QUIN may be attenuating the other excitotoxic effects of L-tryptophan, an antagonist of glutamate receptors may con-

Macrophages convert [$^{13}\text{C}_6$]-L-tryptophan to [$^{13}\text{C}_6$]-QUIN, particularly when stimulated with interferon- γ , and the concentrations achieved in the incubation medium (24 μM) exceed those noted in the CSF of HIV-1-infected patients (up to 15 μM ; Heyes et al., 1992b; Brew and Heyes, unpublished observations). This observation demonstrates that macrophages contain the enzymes necessary to convert L-tryptophan to QUIN. Consequently, it is likely that the activity of other enzymes of the kynurenine pathway are also increased following intracerebral immune activation and macrophage infiltration. Other cells may also convert precursors to QUIN, including astrocytes, which contain 3-hydroxyanthranilate-3,4-dioxygenase (Okuno et al., 1987). The accumulation of QUIN may also reflect the relatively low activity of quinolinic acid phosphoribosyltransferase, the degradation enzyme for QUIN (Foster et al., 1985).

Both indoleamine-2,3-dioxygenase and GTP cyclohydrolase I activity are increased by interferon- γ , tumor necrosis factor- α and other cytokines in macrophages and other cell types (Fig. 1; Niedewieser et al., 1986; Bianchi et al., 1988; Fuchs et al., 1988; Heyes et al., 1992b). Therefore, strong inter-correlations between QUIN, KYNA, L-tryptophan and L-kynurenine with neopterin, β_2 -microglobulin and IgG concentrations in the CSF support a link between indoleamine-2,3-dioxygenase induction with intrathecal inflammatory responses (Table 1; Fuchs et al., 1990; Heyes et al., 1991b, 1992a). These correlations also suggest increased interferon- γ activity within the central nervous system (Griffin et al., 1991). There was minimal evidence that the group increases in CSF QUIN, KYNA or L-kynurenine could be attributed to disruption of the blood-brain barrier. Similar conclusions have been drawn regarding the source of elevated neopterin and β_2 -microglobulin in CSF (Brew et al., 1989, 1990; Griffin et al., 1991).

Dietary L-tryptophan intake was not regulated or quantified in the present study and we cannot exclude the possibility that at least some of the reductions in serum and CSF L-tryptophan concentrations were diet-dependent. However, reduced L-tryptophan intake would be expected to either decrease not only L-tryptophan levels but

also L-kynurenine, KYNA and QUIN concentrations. The reductions in CSF L-tryptophan levels were independent of blood L-tryptophan concentrations, and indicate that the central and systemic L-tryptophan compartments are influenced separately, such as by different local indoleamine-2,3-dioxygenase activities in central nervous system and systemic tissues. The uptake of L-tryptophan into the brain may have also been influenced by changes in the concentrations of large neutral amino acids in the blood of HIV-1-infected patients (Fernstrom, 1983). The levels of large neutral amino acids are reduced in some HIV-1-infected patients (Althoff et al., 1989), which would promote L-tryptophan entry into the CNS (Fernstrom, 1983). Therefore, these observations argue in favor of a role for indoleamine-2,3-dioxygenase induction in accelerating the conversion of L-tryptophan to L-kynurenine, KYNA and QUIN. Depletion of L-tryptophan may reduce the synthesis of serotonin and other indoleamines (Larsson et al., 1989; Heyes et al., 1990a), as well as interfere with the metabolism of protein in both systemic and central nervous system tissues.

While it is clear that induction of indoleamine-2,3-dioxygenase, the depletion of L-tryptophan and increased substrate flux through the kynurenine pathway are associated with immune activation, the reason for this response remains to be established. The magnitude of the increases in kynurenine pathway metabolism, particularly within the central nervous system, and the widespread circumstances in which it occurs, indicate that the reasons and consequences are not trivial. There are arguments that such responses may be both beneficial as well as detrimental. On the positive side, studies in vitro have suggested that activation of indoleamine-2,3-dioxygenase and depletion of intracellular L-tryptophan may be one mechanism by which interferon- γ exerts anti-microbial and anti-proliferative effects on some intracellular parasites and tumor cells (Pfefferkorn and Guyre, 1984; Byrne et al., 1986; Takikawa et al., 1988), but not on others (Turco and Winkler, 1986; Takikawa et al., 1988). Also, the reactions catalysed by indoleamine-2,3-dioxygenase metabolize potentially toxic oxygen-free radicals (Daley-Yates et al., 1988; Siesjo et

al., 1989; Sono, 1989). Conversely, depletion of L-tryptophan may impair protein synthesis and indoleamine metabolism. The production of potentially neurotoxic kynurenine pathway metabolites, including QUIN, L-kynurenine and KYNA, may be another detrimental consequence of indoleamine-2,3-dioxygenase induction. At this point in time, it is not possible to state where the balance between beneficial versus detrimental consequences lies.

Indoleamine-2,3-dioxygenase induction and production of kynurenine pathway metabolites occur in a wide spectrum of immune stimulation (Pfefferkorn and Guyre, 1984; Byrne et al., 1986; Takikawa et al., 1986; Werner et al., 1987, 1989; Bianchi et al., 1988; Heyes and Lackner, 1990; Heyes et al., 1988, 1990b, 1992; Saito et al., 1991a, b). In view of the neuroactive nature of kynurenine pathway metabolites, we propose that such compounds may be final common mediators of neuronal dysfunction and death in inflammatory neurologic disease. This disruption would include functions mediated via N-methyl-D-aspartate receptors, such as learning, memory and synaptic plasticity (Morris et al., 1987). Therefore, strategies to attenuate the neurotoxic effects of QUIN (without disrupting N-methyl-D-aspartate receptor function), or reducing the synthesis of neuroactive kynurenine pathway metabolites, may offer new approaches to therapy of the neurologic deficits associated with HIV-1 infection. Notably, such strategies may also be of benefit in other inflammatory neurologic diseases.

Acknowledgements

We appreciate the assistance of M. Paul and H. Gallardo. This study was supported in part by US Public Health Service Research Grant NS-25701, a grant from the Rudin Foundation, US Army Medical R & D Command project 87PP7856, the Henry M. Jackson Foundation and the Walter Reed Retrovirus Research Group.

References

- Althoff, P.-H., Schifferdecker, E., Forster, H., Michels, B., Hunold, P., St. Klauke, A., Flakenbach, E. and Helm, K.

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r. H., Michels. B., h. E. and Helm. K.

- (1989) Imbalance of the amino acids pattern in patients with AIDS — special treatment with adapted amino acid solution? Fifth International Conference of AIDS, Montreal Abstr. No. Th.B.O. 42., 218.
- Bianchi, M., Bertini, R. and Ghezzi, P. (1988) Induction of indoleamine dioxygenase by interferon in mice: a study with different recombinant interferons and various cytokines. *Biochem. Biophys. Res. Commun.* 152, 237–242.
- Boegman, R.J., Jhamandas, K. and Beninger, R.J. (1990) Neurotoxicity of tryptophan metabolites. *Ann. N.Y. Acad. Sci.* 585, 261–273.
- Brew, B.J., Bhalla, R.B., Fleisher, M., Paul, M., Khan, A., Schwartz, M.K. and Price, R.W. (1989) Cerebrospinal fluid B2 microglobulin in patients infected with human immunodeficiency virus. *Neurology* 39, 830–834.
- Brew, B.J., Bhalla, R.B., Paul, M., Gallardo, H., McArthur, J.C., Schwartz, M.K. and Price, R.W. (1990) Cerebrospinal fluid neopterin in human immunodeficiency virus type-1 infection. *Ann. Neurol.* 28, 556–560.
- Byrne, G., Lehmann, L. and Landry, G. (1986) Induction of tryptophan catabolism is the mechanism for gamma-interferon-mediated inhibition of intracellular *Chlamydia psittaci* replication in T24 cells. *Infect. Immun.* 53, 347–351.
- Daley-Yates, P.T., Powell, A.P. and Smith, L.L. (1988) Pulmonary indoleamine 2,3-dioxygenase activity and its significance in the responses of rats, mice, and rabbits to oxidative stress. *Toxicol. Appl. Pharmacol.* 96, 222–232.
- Feldman, D.S., Gagnon, J., Hofmann, R. and Simpson, J. (1987) Statview II. Abacus Concepts, Berkeley, CA.
- Fernstrom, J.D. (1983) Role of precursor availability in control of monoamine biosynthesis in brain. *Physiol. Rev.* 63, 484–546.
- Foster, A.C., Vezzani, A., French, E.D. and Schwarcz, R. (1984) Kynurenic acid blocks neurotoxicity and seizures induced in rats by the related brain metabolite quinolinic acid. *Neurosci. Lett.* 48, 273–278.
- Foster, A., Whetsell, W.O., Bird, E. and Schwarcz, R. (1985) Quinolinic acid phosphoribosyltransferase in human and rat brain: Activity in Huntington's disease and in quinolinic acid-lesioned rat striatum. *Brain Res.* 336, 207–214.
- Fuchs, D., Hausen, A., Reibnegger, G., Werner, E.R., Dierich, M.P. and Wachter, H. (1988) Neopterin as a marker for activated cell-mediated immunity: application in HIV infection. *Immunol. Today* 9, 150–155.
- Fuchs, D., Moller, A.A., Reibnegger, G., Stockle, E., Werner, E.R. and Wachter, H. (1990) Decreased serum tryptophan in patients with HIV-1 infection correlates with increased serum neopterin and with neurologic/psychiatric symptoms. *J. Acquir. Immune Defic. Syndr.* 3, 873–876.
- Gal, E.M. and Sherman, A.D. (1980) L-Kynurenine: Its synthesis and possible regulatory function in brain. *Neurochem. Res.* 5, 223–239.
- Giuliani, D., Vaca, K. and Noonan, C.A. (1990) Secretion of neurotoxins by mononuclear phagocytes infected with HIV-1. *Science* 250, 1593–1596.
- Griffin, D.E., McArthur, J.C. and Cornblath, D.R. (1991) Neopterin and interferon-gamma in serum and cerebrospinal fluid of patients with HIV-associated neurologic disease. *Neurology* 41, 69–74.
- Halperin, J.J. and Heyes, M.P. (1992) Neuroactive kynurenines in Lyme borreliosis. *Neurology* 42, 43–50.
- Heyes, M.P. and Lackner, A. (1990) Increased cerebrospinal fluid quinolinic acid, kynurenic acid and L-kynurenine in acute septicemia. *J. Neurochem.* 55, 338–341.
- Heyes, M.P. and Markey, S.P. (1988) Quantification of quinolinic acid in rat brain, whole blood and plasma by gas chromatography and negative chemical ionization mass spectrometry: Effects of systemic L-tryptophan administration on brain and blood quinolinic acid concentrations. *Anal. Biochem.* 174, 349–359.
- Heyes, M.P. and Quearry, B.J. (1990) Quantification of kynurenic acid in cerebrospinal fluid: effects of systemic and central L-kynurenine administration. *J. Chromatogr.* 530, 108–115.
- Heyes, M.P., Kim, P. and Markey, S.P. (1988) Systemic lipopolysaccharide and pokeweed mitogen increase quinolinic acid content of mouse cerebral cortex. *J. Neurochem.* 51, 1946–1948.
- Heyes, M.P., Rubinow, D., Lane, C. and Markey, S.P. (1989) Cerebrospinal fluid quinolinic acid concentrations are increased in acquired immune deficiency syndrome. *Ann. Neurol.* 26, 275–277.
- Heyes, M.P., Mefford, I.N., Quearry, B.J., Dedhia, M. and Lackner, A.A. (1990a) Increased ratio of quinolinic acid to kynurenic acid in cerebrospinal fluid of D-retrovirus infected rhesus macaques: Relationship to clinical and viral status. *Ann. Neurol.* 27, 666–675.
- Heyes, M.P., Saito, K., Gravell, M., Jordan, E.K., Lackner, A., Smith, M. and Markey, S.P. (1990b) Increased ratio of quinolinic acid to kynurenic acid and increased indoleamine-2,3-dioxygenase activity in both SRV-D and SIV-infected macaques. *Soc. Neurosci.* 16 (Abstract No. 128.12) 289.
- Heyes, M.P., Brew, B.J., Martin, A., Price, R.W., Salazar, A.M., Sidtis, J.J., Yerger, J.A., Mouradian, M.M., Sadler, A.E., Keilp, J., Rubinow, D. and Markey, S.P. (1991a) Quinolinic acid in cerebrospinal fluid and serum in HIV-1 infection: relationship to clinical and neurologic status. *Ann. Neurol.* 29, 202–209.
- Heyes, M.P., Lackner, A., Kaufman, S. and Milstien, S. (1991b) Cerebrospinal fluid and serum neopterin and biopterin in D-retrovirus-infected rhesus macaques (*Macaca mulatta*): relationship to clinical and viral status. *Aids* 5, 555–560.
- Heyes, M.P., Jordan, E.K., Lee, K., Saito, K., Frank, J.A., Snoy, P.J., Markey, S.P. and Gravell, M. (1992a) Relationship of neurologic status in macaques infected with the simian immunodeficiency virus to cerebrospinal fluid and serum quinolinic acid and kynurenic acid. *Brain Res.* 570, 237–250.
- Heyes, M.P., Saito, K. and Markey, S.P. (1992b) Human macrophages convert L-tryptophan to the neurotoxin quinolinic acid. *Biochem. J.* 283, 633–635.
- Hollander, H. and Stringari, S. (1987) Human immunodeficiency virus-associated meningitis: Clinical course and correlations. *Am. J. Med.* 83, 813–816.
- Holmes, E.W. (1988) Determination of serum kynurenine and

- hepatic tryptophan dioxygenase activity by high-performance liquid chromatography. *Anal. Biochem.* 172, 518-525.
- Larsson, M., Hagberg, L., Norkrans, G. and Forsman, A. (1989) Indole amine deficiency in blood and cerebrospinal fluid from patients with human immunodeficiency virus infection. *J. Neurosci. Res.* 23, 441-446.
- Lipton, S.A., Sucher, N.J., Kaiser, P.K. and Dreyer, E.B. (1991) Synergistic effects of HIV coat protein and NMDA-receptor mediated neurotoxicity. *Neuron* 7, 111-118.
- Morris, R.G., Hagan, J.J., Nadel, L., Jensen, J., Baudry, M. and Lynch, G.S. (1987) Spatial learning in the rat: impairment induced by the thiol-proteinase inhibitor, leupeptin, and an analysis of [3 H]glutamate receptor binding in relation to learning. *Behav. Neural. Biol.* 47, 333-345.
- Mouradian, M.M., Heyes, M.P., Pan, J.-B., Heuser, I.J.E., Markey, S.P. and Chase, T.N. (1989) No changes in central quinolinic acid levels in Alzheimer's disease. *Neurosci. Lett.* 105, 233-238.
- Niederwieser, A., Joller, P., Seger, R., Blau, N., Prader, A., Bettex, J.D., Luthy, R., Hirschel, B., Schaedelin, J. and Vetter, U. (1986) Neopterin in AIDS, other immunodeficiencies, and bacterial and viral infections. *Klin. Wochenschr.* 64, 333-337.
- Okuno, E., Kohler, C. and Schwarcz, R. (1987) Rat 3-hydroxyanthranilic acid oxygenase: Purification from the liver and immunocytochemical localization in the brain. *J. Neurochem.* 49, 771-780.
- Pfefferkorn, E.R. and Guyre, P.M. (1984) Inhibition of growth of *Toxoplasma gondii* in cultured fibroblasts by human recombinant gamma interferon. *Infect. Immun.* 44, 211-216.
- Redfield, R.R., Wright, D.C. and Tramont, E.C. (1986) The Walter Reed staging classification for HTLV-III/LAV infection. *N. Engl. J. Med.* 314, 131-132.
- Saito, K., Lackner, A., Markey, S.P. and Heyes, M.P. (1991a) Cerebral cortex and lung indoleamine-2,3-dioxygenase activity is increased in type-D retrovirus infected macaques. *Brain Res.* 540, 353-356.
- Saito, K., Markey, S.P. and Heyes, M.P. (1991b) Chronic effects of gamma-interferon on quinolinic acid and indoleamine-2,3-dioxygenase in brain of C57BL6 mice. *Brain Res.* 546, 151-154.
- Siesjo, B.K., Agardh, C.D. and Bengtsson, F. (1989) Free radicals and brain damage. *Cerebrovasc. Brain. Metab. Rev.* 1, 165-211.
- Sono, M. (1989) The roles of superoxide anion and methylene blue in the reductive activation of indoleamine-2,3-dioxygenase by ascorbic acid or by xanthine oxidase-hypoxanthine. *J. Biol. Chem.* 264, 1616-1622.
- Takikawa, O., Yoshida, R., Kido, R. and Hayaishi, O. (1986) Tryptophan degradation in mice initiated by indoleamine-2,3-dioxygenase. *J. Biol. Chem.* 261, 3648-3653.
- Takikawa, O., Kuroiwa, T., Yamazaki, F. and Kido, R. (1988) Mechanism of interferon-gamma action. Characterization of indoleamine 2,3-dioxygenase in cultured human cells induced by interferon-gamma and evaluation of the enzyme-mediated tryptophan degradation in its anticellular activity. *J. Biol. Chem.* 263, 2041-2048.
- Turco, J. and Winkler, H.H. (1986) Gamma-interferon-induced inhibition of the growth of *Rickettsia prowazekii* in fibroblasts cannot be explained by the degradation of tryptophan or other amino acids. *Infect. Immun.* 53, 38-46.
- Werner, E.R., Bitterlich, G., Fuchs, D., Hausen, A., Reibnegger, G., Szabo, G., Dierich, M.P. and Wachter, H. (1987) Human macrophages degrade tryptophan upon induction by interferon-gamma. *Life Sci.* 41, 273-280.
- Werner, E.R., Werner-Felmayer, G., Fuchs, D., Hausen, A., Reibnegger, G. and Wachter, H. (1989) Parallel induction of tetrahydrobiopterin biosynthesis and indoleamine-2,3-dioxygenase activity in human cells and cell lines by γ -interferon. *Biochem. J.* 262, 861-866.
- Whetsell, W.O. and Schwarcz, R. (1989) Prolonged exposure to submicromolar concentrations of quinolinic acid causes excitotoxic damage in organotypic cultures of rat corticostriatal system. *Neurosci. Lett.* 97, 271-275.

Key words: Antibody response

Summary

The magnitude of the antibody response via cerebral immunized rats: immunization immunogenicity of the antibody CNS, since ov cervical node:

Introduction

Previous studies have shown that antigens yield introduced into conventional animals, 1965; Saito et al. (1991) reported that the contribution of CSF-antigen identified. N antigens will

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Changes in Plasma Amino Acid Concentrations in Response to HIV-1 Infection

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Plasma concentrations of 21 amino acids were determined for 20 control subjects and 20 subjects infected with human immunodeficiency virus type 1 (HIV). Compared with the control subjects, the HIV-infected group had lower cystine, tryptophan, and methionine (decreased 67%, 52%, and 32%, respectively, $P < 0.001$ for each) and increased taurine (230%, $P < 0.001$) and lysine concentrations (30%, $P < 0.001$). Other amino acid concentrations changed modestly. Amounts of cystine, tryptophan, methionine, taurine, and lysine did not differ significantly between subgroups of HIV-infected subjects with >200 ($n = 6$) or <200 ($n = 14$) $CD4^+$ lymphocytes per microliter, suggesting that the concentrations decrease soon after infection and change little thereafter. Activation of metabolism of cystine to taurine may explain reciprocal changes in these amino acids and known depletion of cystine and glutathione. The selective changes in amino acid profiles observed during HIV infection differ from those recognized for malnutrition or other pathological processes.

Indexing Terms: tryptophan/cystine/taurine/metabolism/nutrition

Infection with human immunodeficiency virus type 1 (HIV-1) triggers a range of metabolic changes in addition to the progressive deficits in cellular immunity and increased susceptibility to opportunistic infections that are its clinical hallmarks, and the progression to the acquired immunodeficiency syndrome (AIDS) (1, 2). The most tangible metabolic response to HIV infection is weight loss, due to a combination of factors including increased metabolic rate, anorexia, and malabsorption (3-10). Not infrequently, this weight loss progresses to a severe wasting syndrome (3, 8). There are also multiple, more subtle metabolic changes that can be measured: Early in HIV infection the resting metabolic rate increases (3-8), as do rates of protein turnover (3, 9). Concentrations of the adrenal steroid hormone dehydroepiandrosterone sulfate decrease (11), the anion gap increases (12), and disturbances in lipid metabolism produce decreased serum cholesterol and increased triglycerides (13). Plasma cystine and tryptophan (14-18) and intracellular glutathione concentrations also decrease (14-16).

The diverse metabolic responses to HIV infection may contribute to the pathophysiology of the disease. The

increased metabolic rate and protein turnover contribute to weight loss and muscle wasting. The decrease in cystine, which usually is the rate-limiting substrate for the synthesis of glutathione (14-16, 19), may be a factor in triggering the replication of HIV. Other findings indicate that intracellular thiols, such as glutathione, suppress HIV replication (20, 21). These results have led to *N*-acetylcysteine or other compounds that might boost glutathione concentrations being considered for therapy for HIV infection. Decreased availability of tryptophan may contribute to neuropsychiatric disease in HIV infection by decreasing production of the neurotransmitter serotonin, promoting affective disorders (17, 18). Also, tryptophan deprivation serves as a physiological defense mechanism against some intracellular parasites such as *Toxoplasma gondii* and *Chlamydia* species (22), and in this respect could be an adaptive response against infection.

The basis for the complex metabolic responses to HIV infection is not fully understood, but it is likely to be mediated by lymphokines. Concentrations of many lymphokines such as γ -interferon change dramatically after HIV infection (23, 24). γ -Interferon increases expression of indoleamine-2,3-dioxygenase, an enzyme that cleaves the indole ring of tryptophan (25-27). Induction of this enzyme by lymphokine responses to HIV infection may cause the decreased plasma concentrations of tryptophan (17, 18); there are concurrent increases in the concentrations of end products of tryptophan metabolism such as quinolinic acid (28, 29).

Although major changes in tryptophan and cystine concentrations are known to occur in HIV infection, there is limited information regarding other amino acids. Rather than a specific response to lymphokines, the decreases in cystine and tryptophan may represent a general depletion of essential amino acids analogous to that seen in starvation. Here we examined the concentrations of 21 plasma amino acids during HIV infection and compared their profiles in this disease with those during other pathological processes. An extensive database is available for comparison of amino acid profiles that have been characterized for many physiological and pathological conditions such as liver disease (30), starvation (31), pellagra (32), chronic pulmonary disease (33), posttraumatic injury (34), and various types of cancer (35, 36).

Materials and Methods

HIV infection of subjects was confirmed by testing for antibodies to HIV antigens and by confirmatory testing with Western blot analysis as part of standard protocols for enrollment in studies of the AIDS Clinical Trial Unit at Washington University. Protocols for enrollment of

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Received November 22, 1993; accepted February 14, 1994.

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subjects and collection of specimens were approved by the Institutional Review Board at Washington University. Heparinized blood was collected from HIV-infected subjects during their routine visits for enumeration of CD4 lymphocytes. A portion of the whole-blood sample was used for flow cytometric analysis, and a portion was centrifuged for 15 min at 2000g to yield plasma for amino acid analysis. Samples were prepared for flow cytometry with the Q-Prep whole-blood lysis technique involving antibodies, lysis reagents, and equipment from Coulter Corp. (Hialeah, FL). We performed five analyses on each sample, using double antibody-labeling and a Profile I flow cytometer according to recently described guidelines for lymphocyte analysis (37). The proportion of CD4⁺ lymphocytes was determined by using reagents from Coulter with an antibody pair consisting of fluorescein isothiocyanate-conjugated antibody to CD3 and a phycoerythrin-conjugated antibody to CD4. Other antibody pairs were for gating markers, isotypic controls, CD8 counts, and B-cell counts. The total lymphocyte count was determined with a Coulter Model S counter by using a blood specimen collected concomitantly with other samples into a tube containing potassium EDTA. Plasma samples for amino acid analysis from HIV-infected subjects were selected so as to obtain a broad distribution of CD4 counts. Selection of samples was otherwise random, and samples represented a broad range of age, clinical status classified according to revised criteria of the Centers for Disease Control and Prevention (38), and treatment protocols as listed in Table 1. All but one sample were from males, despite active recruitment of female subjects. Control samples were collected from volunteers who by history were in good health and did not have unexplained weight loss. The age and gender distribution of the control subjects were similar to those of the experimental group. The control group had a mean age of 35 ± 10 years, vs 36 ± 10 for the experimental group.

Plasma samples for amino acid analysis were stored frozen at -20°C and prepared for analysis by addition of norleucine as an internal standard and of sulfosalicylic acid to bring the sample to a concentration of 6 g/L. After centrifugation, the pH of the supernatant was adjusted and 5-μL samples were analyzed on a Beckman 6300 analyzer (Beckman Instruments, Fullerton, CA) by postcolumn reaction with o-phthalaldehyde and fluorescence detection. Calibrators were run daily. The system does not detect secondary amino acids such as proline, hydroxyproline, and sarcosine. Values for aspartic acid are not reported, because they were below the limits of detection in many subjects. Concentrations of cystine and tryptophan in some subjects with HIV infection were below the lower limits of accurate quantification by the analyzer, which corresponded to a sample concentration of 6 μmol/L. In these analyses, values of 6 μmol/L were assigned for these amino acids for the purpose of calculating group means and standard deviations. This applied to eight samples for cystine and two samples for tryptophan, all from HIV-infected subjects. The significance of differences between group means

Table 1. Characteristics of the HIV-infected subjects.

Age	Race	CD4 count	CDC stage*	Therapy
36	White	49	C3	AZT, DDI, Dapsone
29	Black	70	B3	DDI, TMP/SMX
28	White	476	B2	Blinded antiretroviral
20 ^b	Black	260	A2	Blinded antiretroviral
46	White	28	B3	AZT, pentamidine aerosol
36	White	15	B3	DDI, pentamidine aerosol
34	White	13	C3	AZT, TMP/SMX
48	White	195	A3	None
17	Black	166	B3	AZT, DDI, TMP/SMX
32	White	31	C3	DDI, pentamidine aerosol
48	White	314	A2	Blinded antiretroviral
33	Black	13	C3	AZT, TMP/SMX
50	White	188	A3	TMP/SMX
48	White	39	C3	AZT, α-interferon
38	White	451	A2	Blinded antiretroviral
30	Black	107	A3	AZT, DDI, pentamidine aerosol
31	White	301	A2	None
53	White	41	B3	AZT, DDC, TMP/SMX
40	White	27	C3	DDI, TMP/SMX
30	Black	296	A2	AZT

* CDC (Centers for Disease Control and Prevention) stage is defined by CD4 count: A, >500; B, 200-499; C, <200; and by symptoms: stage 1, asymptomatic or acute HIV infection; stage 2, symptomatic without AIDS-defining infections; stage 3, symptomatic with AIDS-defining illness, which include *Pneumocystis carinii* pneumonia, toxoplasmosis of the brain, *Mycobacterium avium* complex infection, extrapulmonary cryptococcosis or histoplasmosis, primary lymphoma of the brain, Kaposi sarcoma, and others listed in new criteria for AIDS (38).

^b Female patient. All other patients were male.

AZT, 3'-azidothymidine or zidovudine; DDI, 2',3'-dideoxyinosine or didanosine; DDC, 2',3'-dideoxycytosine; TMP/SMX, trimethoprim and sulfamethoxazole.

was assessed as a two-tailed probability with Student's *t*-test. Correlations of amino acid concentrations with CD4 counts were analyzed with InPlot software (Graph-Pad, San Diego, CA) and assessed with a two-tailed *t*-test. Considering that *t*-tests were performed for 20 sets of data corresponding to results for each of 20 different amino acids, there is ~ a 20-fold increased probability of noting a difference that had occurred by chance. For this reason, the Bonferroni correction (division of usual level of statistical significance, *P* = 0.05, by the number of comparisons) was applied and only differences with *P* < 0.0025 were considered statistically significant.

Results

The mean and standard deviations of plasma concentrations of 21 different amino acids in controls and subjects with HIV-1 infection are compared in Table 2. Results for glutamic acid and glutamine (Glu + Gln) were combined, because the samples were not processed immediately to prevent significant conversion of glutamine to glutamic acid. Mean concentrations of several essential amino acids were dramatically decreased in the HIV-infected group. Cystine decreased by 67%, tryptophan by 52%, and methionine by 32%. Other amino acids showed substantial increases, including taurine,

subjects

Table 2. Plasma amino acid concentrations.

		Mean \pm SD, $\mu\text{mol/L}$		
Amino acid		Controls (n = 20)	HIV-infected (n = 20)	Change, %
Cystine		46 \pm 20	15 \pm 13 ^a	-67
Tryptophan		46 \pm 22	22 \pm 12 ^a	-52
Methionine		28 \pm 8	19 \pm 6 ^a	-32
Asparagine		45 \pm 22	34 \pm 10	-24
Tyrosine		65 \pm 18	50 \pm 13	-23
Arginine		67 \pm 27	58 \pm 16	-13
Threonine		134 \pm 41	121 \pm 34	-10
Isoleucine		69 \pm 20	64 \pm 15	-7
Leucine		131 \pm 31	123 \pm 28	-6
Valine		216 \pm 33	208 \pm 43	-4
Glu + Gln		598 \pm 105	595 \pm 87	-1
Alanine		398 \pm 92	406 \pm 76	+2
Phenylalanine		56 \pm 16	57 \pm 14	+2
Histidine		71 \pm 6	74 \pm 15	+4
γ -Aminobutyric acid		15 \pm 10	17 \pm 10	+13
Serine		103 \pm 26	117 \pm 25	+14
Glycine		207 \pm 49	255 \pm 49	+23
Ornithine		94 \pm 22	121 \pm 38	+29
Lysine		173 \pm 35	225 \pm 37 ^a	+30
Taurine		47 \pm 11	155 \pm 63 ^a	+230

^a Statistically significant at $P < 0.001$.

which increased by 230%, and lysine, by 30%. All of these differences were significant ($P < 0.0025$), as indicated in Table 1. Amounts of most amino acids were not significantly changed. Concentrations of several essential amino acids such as leucine, isoleucine, valine, threonine, and phenylalanine were similar in experimental and control groups.

Comparison of taurine concentrations for the two subject groups (Fig. 1A) shows a marked increase in HIV-infected subjects, with almost complete discrimination between the two groups. There is overlap of only a single data point. Although there were substantial differences in the cystine (Fig. 1B) and tryptophan concentrations (Fig. 1C) between the two groups, there was greater variation among individuals and more overlap between HIV-infected and control subjects. Samples from control and HIV-infected groups were not obtained specifically from fasting subjects; this may increase experimental variability in the present study.

The relation between amino acid concentrations and the number of CD4⁺ lymphocytes during the course of HIV infection was examined. The depletion of CD4⁺ lymphocytes correlates with clinical progression of HIV infection, and a decrease in the CD4 count to < 200 per microliter has been added recently as a criterion for establishing the diagnosis of AIDS in patients with HIV infection (1, 37). Table 3 presents a comparison of amino acid concentrations in subgroups of HIV-infected individuals with CD4 counts < 200 or > 200 . No significant differences ($P < 0.0025$) were noted for any amino acids between the two subgroups, although asparagine, Glu + Gln, and phenylalanine were increased 29%, 19%, and 33%, respectively, and mean concentrations of cystine

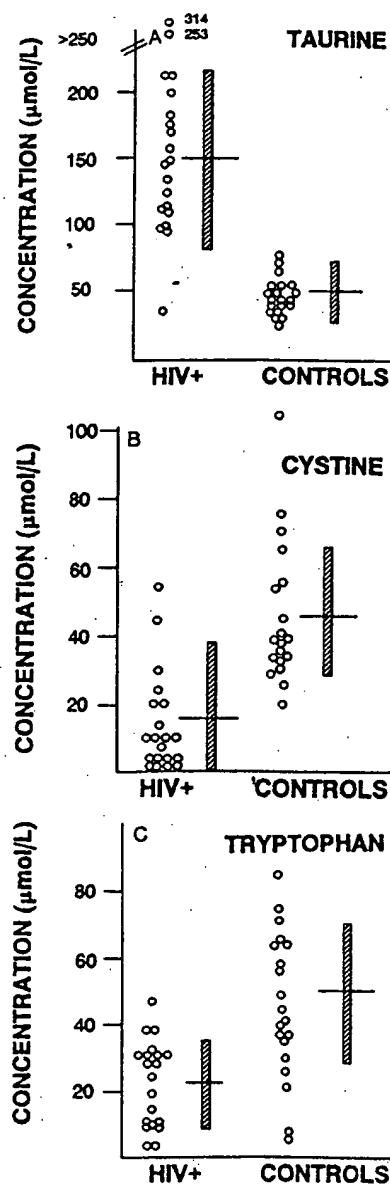


Fig. 1. Comparison of taurine (A), cystine (B), and tryptophan (C) concentrations in plasma of HIV-infected subjects and controls. Bars adjacent to data points indicate mean and SD.

were 53% less and tryptophan 23% less in the low-CD4-count subgroup. Concentrations of leucine, lysine, isoleucine, valine, methionine, and threonine were unchanged or slightly increased in subjects with low CD4 counts. Comparison of the subgroups with a CD4 count $< \text{or} > 200$ indicated that progression of HIV infection to AIDS was not clearly associated with progressive depletion of any amino acid. In fact, amounts of some essential amino acids, such as phenylalanine, sometimes increased during the course of HIV infection. Amino acids in the eight subjects who were clinically asymptomatic (see Table 1) showed markedly different concentrations compared with the controls, similar to those of the total HIV-infected group. For the asymptomatic subgroup of HIV-infected subjects, the concentration of cystine was 19 ± 14 mmol/L (a 59% decrease vs controls), tryptophan was 22 ± 10 mmol/L (a 52% decrease), and taurine was 169 ± 68 (a 260% increase).

Table 3. Amino acid concentrations for HIV-infected subgroups.

Amino acid	Mean \pm SD, μ mol/L	
	CD4 <200 (n = 14)	CD4 >200 (n = 6)
Cystine	12 \pm 12	20 \pm 16
Tryptophan	21 \pm 13	26 \pm 9
Methionine	20 \pm 7	17 \pm 2
Asparagine	36 \pm 4	28 \pm 4
Tyrosine	52 \pm 15	47 \pm 8
Arginine	62 \pm 17	50 \pm 11
Threonine	121 \pm 37	121 \pm 38
Isoleucine	65 \pm 17	60 \pm 10
Leucine	127 \pm 31	114 \pm 17
Valine	215 \pm 48	191 \pm 26
Glu + Gln	624 \pm 80	527 \pm 63
Alanine	423 \pm 91	365 \pm 91
Phenylalanine	61 \pm 14	46 \pm 5
Histidine	75 \pm 17	74 \pm 10
α -Aminobutyric acid	19 \pm 11	14 \pm 6
Serine	121 \pm 18	107 \pm 18
Glycine	265 \pm 46	232 \pm 46
Ornithine	126 \pm 33	109 \pm 49
Lysine	231 \pm 42	211 \pm 15
Taurine	148 \pm 55	172 \pm 81

No significant differences at $P < 0.0025$.

Changes in amino acid concentrations during the progression of HIV infection also were assessed by examining the correlation between amino acid concentrations and CD4 counts and treating the correlation as a continuous variable (plots not shown). At low CD4 counts, there were trends of increasing phenylalanine ($P < 0.02$) and Glu + Gln ($P < 0.03$), and trends of decreasing tryptophan and cystine (both $P > 0.05$). However, none of these relations reached the threshold of $P < 0.0025$ for significance of multiple comparisons.

Discussion

Individuals with HIV infection have diminished plasma concentrations of tryptophan and cystine (14-18). Data presented here indicate that depletions of these amino acids is highly specific. The only other amino acid observed to undergo a major decrease is methionine (though to a lesser extent than cystine), and loss of this amino acid may be explained by metabolic interconversion of cysteine and methionine (39).

HIV infection produced relatively little change in the concentrations of essential amino acids such as threonine, valine, isoleucine, leucine, and phenylalanine. In starvation, the concentrations of these amino acids as well as cystine and tryptophan are depleted relatively uniformly (31). Individuals with HIV infection have many factors contributing to malnutrition such as anorexia, intestinal malabsorption, intestinal parasitemia, and increased metabolic demands from infection (3-10). These factors contribute to decreases in the concentrations of many nutrients such as vitamins A, E, riboflavin, B₆, and B₁₂, and copper and zinc (40), but our

results indicate that general deficiencies of essential amino acids do not develop. The highly selective decrease of cystine and tryptophan in HIV-infected individuals indicates that these amino acids are not diminished because of a nutritional deficit. Specific pathways for the uptake or metabolism of cystine and tryptophan must be activated. A likely pathway for the consumption of tryptophan is via induction of the synthesis of indoleamine-2,3-dioxygenase (17, 18). Our finding of increased concentrations of taurine in HIV-infected subjects supports analogous consumption of cysteine via conversion to taurine, which can occur by two parallel pathways that are initiated by cysteine dioxygenase and cysteine decarboxylase, respectively. The pathway initiated by conversion of cysteine to cysteine sulfinic acid by cysteine dioxygenase is usually the predominant route, and activity of this enzyme is rate-limiting for the pathway (39, 41).

Previous work (17) and the data presented here provide evidence that decreases in plasma concentrations of tryptophan, cystine, and methionine occur relatively early in the course of HIV infection, well before development of clinically defined AIDS. These amino acids were already severely depleted in the subgroup of patients with CD4 counts >200; therefore there is a relatively weak trend of tryptophan and cystine relative to CD4 counts—most of the change had occurred earlier in the course of HIV infection. This is expected if the selective decreases in amino acid concentrations are mediated by changes in amounts of cytokines such as γ -interferon. Cytokine responses develop rapidly after HIV infection, even before development of antibody responses to HIV antigens (1, 23, 24). Not only were changes in amino acid concentrations not correlated with CD4 counts, but they also were not correlated with clinical staging of disease. Eight clinically asymptomatic subjects with HIV infection appeared to have marked changes in amino acid concentrations with respect to controls. Therapy of subjects with HIV infection is not considered a likely source of difference from controls because, as listed in Table 1, treatment of the HIV-infected subjects was with a variety of medications and two of the subjects received no medications.

The highly specific changes in the pattern of amino acid concentrations in HIV infection do not correspond to recognized patterns occurring in other pathological processes. Complete starvation or severe protein-calorie malnutrition results in a relatively symmetric reduction in essential amino acids (31). Many disorders such as sepsis, hepatic dysfunction, and advanced emphysema are characterized more by increases in selective amino acids than decreases (30-34). Studies of changes of amino acid concentrations in cancer patients note decreases of different amino acids with several types of malignancy (35). The reported pattern that is most similar to that in patients with HIV infection is that occurring in patients with lung cancer and breast cancer. However, individuals with these cancers appear to have lesser decreases in tryptophan and cystine than do individuals with HIV infection (36). More extensive ana-

analysis of the amino acid patterns in HIV infection may be useful in characterizing the metabolic changes that occur early in infection. It remains to be established whether depletions of cystine and tryptophan are prognostic indicators or whether losses of these amino acids contribute to the pathophysiology of HIV infection. The highly specific responses of cystine and tryptophan metabolism could serve as second messengers or effectors for lymphokine responses.

We thank Sharon Bader for technical assistance. G. L. H. was supported as a Medical Scholar of the Charles E. Culpeper Foundation. The Washington University AIDS Clinical Trial Unit is supported by grant AI-25908 from the NIH.

References

- Pantaleo G, Graziosi C, Fauci AS. The immunopathogenesis of human immunodeficiency virus infection. *New Engl J Med* 1993; 328:327-35.
- Levy JA. The transmission of HIV and factors influencing progression to AIDS. *Am J Med* 1993;95:86-100.
- Grunfeld C, Feingold KR. Metabolic disturbances and wasting in the acquired immunodeficiency syndrome. *N Engl J Med* 1992; 327:329-37.
- Grunfeld C, Pang M, Shimizu JK, Jensen P, Feingold KR. Resting energy expenditure, caloric intake, and short-term weight change in human immunodeficiency virus infection and the acquired immunodeficiency syndrome. *Am J Clin Nutr* 1992;55:455-60.
- Kotler DP, Tierney AR, Brenner SK, Couture S, Wang J, Pierson RN. Preservation of short-term energy balance in clinically stable patients with AIDS. *Am J Clin Nutr* 1990;51:7-13.
- Hommes MJT, Romijn JA, Enderit E, Sauerwein HP. Resting energy expenditure and substrate oxidation in human immunodeficiency virus (HIV)-infected asymptomatic men: HIV affects host metabolism in the early asymptomatic stage. *Am J Clin Nutr* 1991;54:311-5.
- Melchior JC, Salmon D, Rigaud D, Lepout C, Bouvet E, Detruhis P, et al. Resting energy expenditure is increased in stable, malnourished HIV-infected patients. *Am J Clin Nutr* 1991;53:437-41.
- Macallan DC, Griffin GE. Metabolic disturbances in AIDS [Letter]. *N Engl J Med* 1992;327:1530-1.
- Grunfeld C, Kotler DP. Wasting in the acquired immunodeficiency syndrome. *Semin Liver Dis* 1992;12:175-87.
- Stein TP, Nutinsky C, Condoluci D, Schluter MD, Leskiw MJ. Protein and energy substrate metabolism in AIDS patients. *Metabolism* 1990;39:876-81.
- Wisniewsky TL, Hilton CW, Morse EV, Svec F. The relationship of serum DHEA-S and cortisol levels to measures of immune function in human immunodeficiency virus-related illness. *Am J Med Sci* 1993;305:79-83.
- Siucher B, Levinson SS. Human immunodeficiency virus infection and anion gap. *Ann Clin Lab Sci* 1993;23:249-55.
- Shor-Posner G, Basit A, Lu Y, Cabrejos C, Chang J, Fletcher M, et al. Hypocholesterolemia is associated with immune dysfunction in early human immunodeficiency virus-1 infection. *Am J Med* 1993;94:515-9.
- Eck H-P, Grumder H, Harmann M, Petzoldt D, Daniel V, Droge W. Low concentrations of acid-soluble thiol (cysteine) in the blood plasma of HIV-1 infected patients. *Biol Chem Hoppe-Seyler* 1989;370:101-8.
- Buhl R, Jaffe HA, Holroyd KJ, Wells FB, Mastrangeli A, Sakini C, et al. Systemic glutathione deficiency in symptom-free HIV-seropositive individuals. *Lancet* 1989;2:1294-7.
- de Quay B, Malinverni R, Lauterburg BH. Glutathione depletion in HIV-infected patients: role of cysteine deficiency and effect of oral N-acetylcysteine. *AIDS* 1992;6:815-9.
- Werner ER, Fuchs D, Hausen A, Jaeger H, Reibnegger G, Werner-Felmayer G, et al. Tryptophan degradation in patients infected by human immunodeficiency virus. *Biol Chem Hoppe-Seyler* 1988;369:337-40.
- Fuchs D, Moller AA, Reibnegger G, Stockle E, Werner ER, Richter H. Decreased serum tryptophan in patients with HIV-1

- infection correlates with increased serum neopterin and with neurologic/psychiatric symptoms. *J Acquir Immune Defic Syndr* 1990;3:873-6.
- Penttila KE. Role of cysteine and taurine in regulating glutathione synthesis by periportal and perivenous hepatocytes. *Biochem J* 1990;269:659-64.
- Roederer M, Staal FJT, Raju PA, Ela SW, Herzenberg LA, Herzenberg LA. Cytokine-stimulated human immunodeficiency virus replication is inhibited by N-acetyl-L-cysteine. *Proc Natl Acad Sci USA* 1990;87:4884-8.
- Kalebic T, Kinter A, Poli G, Anderson ME, Meister A, Fauci A. Suppression of human immunodeficiency virus expression in chronically infected monocytic cells by glutathione, glutathione ester, and N-acetylcysteine. *Proc Natl Acad Sci USA* 1991;88:986-90.
- Byrne G, Lehmann L, Landry G. Induction of tryptophan catabolism is the mechanism for gamma-interferon-mediated inhibition of intracellular Chlamydia psittaci replication in T24 cells. *Infect Immun* 1986;53:347-51.
- Doweiko JP. Hematologic aspects of HIV infection. *AIDS* 1993;7:753-7.
- Francis ML, Meltzer MS, Gendelman HE. Interferons in the persistence, pathogenesis, and treatment of HIV infection. *AIDS Res Hum Retroviruses* 1991;8:199-207.
- Taylor MW, Feng G. Relationship between interferon- γ , indoleamine-2,3-dioxygenase and tryptophan. *FASEB J* 1991;5: 2516-22.
- Heyes MP, Saito K, Jacobowitz D, Markey SP, Takikawa O, Vickers JH. Poliovirus induces indoleamine-2,3-dioxygenase and quinolinic acid synthesis in macaque brain. *FASEB J* 1992;6: 2977-89.
- Takikawa O, Kuroiwa T, Yamazaki F, Kido R. Mechanism of interferon-gamma action. Characterization of indoleamine 2,3-dioxygenase in cultured human cells induced by interferon-gamma and evaluation of the enzyme-mediated tryptophan degradation in its anticellular activity. *J Biol Chem* 1988;263:2041-8.
- Heyes MP, Saito K, Markey SP. Human macrophages convert L-tryptophan to the neurotoxin quinolinic acid. *Biochem J* 1992; 283:633-5.
- Heyes MP, Rubinow D, Lane C, Markey SP. Cerebrospinal fluid quinolinic acid concentrations are increased in acquired immune deficiency syndrome. *Ann Neurol* 1989;26:275-7.
- Steigmann F, Szanto PB, Poulos A, Lim PE, Dubin A. Significance of serum aminograms in diagnosis and prognosis of liver disease. *J Clin Gastroenterol* 1984;6:453-60.
- Holt LE Jr, Snyderman SE, Norton PM, Roitman E, Finch J. The plasma aminogram in kwashiorkor. *Lancet* 1963;ii:1343-8.
- Truswell AS, Hansen JDL, Wannenburg P. Plasma tryptophan and other amino acids in pellagra. *Am J Clin Nutr* 1968;21:1314-20.
- Hofford JM, Milakofsky L, Vogel WH, Sacher RS, Savage GJ, Pell S. The nutritional status in advanced emphysema associated with chronic bronchitis. *Am Rev Resp Dis* 1990;141:902-8.
- Jeevanandam M, Young DH, Ramias L, Schiller WR. Effect of major trauma on plasma free amino acid concentration in geriatric patients. *Am J Nutr* 1990;51:1040-5.
- Pisters PWT, Brennan MF. Amino acid metabolism in human cancer cachexia. *Ann Rev Nutr* 1990;10:107-32.
- Zhang PC, Pang CP. Plasma amino acid patterns in cancer. *Clin Chem* 1992;38:1198-9.
- Centers for Disease Control and Prevention. Guidelines for the performance of CD4⁺ T-cell determinations in persons with human immunodeficiency virus infection. *MMWR Morb Mortal Wkly Rep* 1992;41(no. RR-8):1-17.
- Centers for Disease Control and Prevention. 1993 revised classification system for HIV infection and expanded surveillance case definition for AIDS among adolescents and adults. *MMWR Morb Mortal Wkly Rep* 1993;41:1-19.
- Stipanuk MH. Metabolism of sulfur-containing amino acids. *Ann Rev Nutr* 1986;6:179-209.
- Beach RS, Mantero-Atienza E, Shor-Posner G, Javier JJ, Szapocznik J, Morgan R, et al. Specific nutrient abnormalities in asymptomatic HIV-1 infection. *AIDS* 1992;6:701-8.
- Tappaz M, Almarghini K, Legay F, Remy A. Taurine biosynthesis enzyme cysteine sulfinate decarboxylase (CSD) from brain: the long and tricky trail to identification. *Neurochem Res* 1992; 17:849-59.

Threonine and Methionine Are Limiting Amino Acids for Protein Synthesis in Patients with AIDS^{1,2,3}

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ABSTRACT This study was conducted to identify the most rate-limiting amino acids for whole-body protein synthesis in acquired immunodeficiency syndrome (AIDS) patients. We postulated that an essential amino acid that would be rate limiting in AIDS should have a low basal plasma concentration and should remain at a low level during amino acid infusion. Seven male AIDS patients (median age 37 y, CD4 cell count: 76 mm⁻³) without any clinically active opportunistic infection during the month before the experiment were infused intravenously with a complete amino acid-glucose mixture for 2.5 h. Eight healthy volunteers were used as controls. Before the infusion, the concentrations of most free essential amino acids (methionine, threonine, histidine, isoleucine, leucine and tryptophan) were significantly lower ($P < 0.05$) in AIDS patients than in controls. Most plasma free essential amino acids increased significantly during infusion. However, the absolute increase above basal levels for threonine, valine, lysine, ($P < 0.05$) and methionine ($P < 0.073$) was smaller in AIDS patients than in control subjects. Thus, threonine and possibly methionine may be rate limiting for whole-body protein synthesis in AIDS patients, suggesting that there are selective amino acid requirements in patients with AIDS. *J. Nutr.* 128: 1342–1348, 1998.

KEY WORDS: • amino acid requirements • protein metabolism • AIDS • limiting amino acids • humans

Infection with the human immunodeficiency virus (HIV)⁵ has a devastating effect on nutritional status. Weight loss, often profound in magnitude, is one of the most universal features of HIV infection, and patients may lose 30–50% of their body mass before succumbing to the disease (Gorbach et al. 1993, Sauerwein 1993). Acquired immunodeficiency syndrome (AIDS) is characterized by a predominant loss of lean tissue (Kotler 1985). Multiple factors in

different combinations contribute to AIDS related malnutrition and increased host requirements; these include anorexia, malabsorption, abnormal utilization and excretion of nutrients. This is correlated with the severity of the HIV infection and with secondary infections. Malnutrition has a deleterious effect on immune function and thus may potentially accelerate the progression of immune deficiency in HIV infection (Chandra 1991).

Understanding the changes in protein and amino acid metabolism in HIV infection is crucial because they underlie the loss of protein (i.e., of lean tissue). Measurements of amino acid kinetics in AIDS patients have shown the characteristic features of a hypermetabolic response with increased protein turnover (Lieberman et al. 1994, Macallan et al. 1995, Stein et al. 1990). In addition, the degradation of tryptophan via the kynurenine pathway is stimulated (Werner et al. 1988), and sulfur amino acids and glutathione metabolism are altered (Buhl et al. 1989, Eck et al. 1989, Hortin et al. 1994).

Undernutrition may contribute to the protein wasting in HIV patients because nutritional support seems to have been beneficial (Boulétreau et al. 1995, Melchior et al. 1996, Sukkar & Giacosa 1995). Short-term parenteral hyperalimentation enriched with amino acids is capable of reversing net protein catabolism (Macallan et al. 1995, Selberg et al. 1995). However, the amino acid requirements for the replenishment of protein mass in adult humans are not known with certainty (Pellert 1990, Reeds et al. 1994). The aim of this study was

¹ Presented in part in abstract form at the first International Meeting on Nutrition-HIV Infection, April 28–29, 1995, Cannes, France [Charrier, S., Laurichesse, H., Tauveron, I., Laveran, H., Thieblot, Ph., Grizard, J., Beytout, J. & Champredon, C. (1995) Effect of amino acid infusion on plasma tryptophan concentrations in HIV-infected patients. Preliminary results. Abstract book 103], at the fifth European Conference on Clinical Aspects and Treatment of HIV Infection, September 26–29, 1995, Copenhagen, Denmark. [Laurichesse, H., Tauveron, I., Gourdon, F., Cormerais, L., Charrier, S., Grizard, J., Thieblot, Ph., Beytout, J. & Champredon, C. (1995) Is threonine a rate limiting amino acid for protein synthesis in AIDS patients? Abstract book 173] and at the XI International Conference on AIDS, July 7–12, 1996, Vancouver, Canada [Champredon, C., Laurichesse, H., Tauveron, I., Gourdon, F., Charrier, S., Cormerais, L., Thieblot, Ph., Grizard, J. & Beytout, J. (1996) Rate limiting amino acids for protein synthesis in AIDS. Abstract book B 3260].

² Supported by a grant from the Agence Nationale de Recherche sur le SIDA, the Direction de la Recherche et des Etudes Doctorales and the Région Auvergne.

³ The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 USC section 1734 solely to indicate this fact.

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⁵ Abbreviations used: AIDS, acquired immunodeficiency syndrome; BW, body weight; HIV, human immunodeficiency virus.

TABLE 1

Characteristics of the acquired immunodeficiency syndrome (AIDS) patients and the control subjects

	Age, y	Height, m	Weight, kg	BMI, kg/m ²	CD4+, mm ⁻³	AIDS-defining events	Current treatment
AIDS patients							
1	30	1.75	48.5	15.8	8	FE, VZ	DDI
2	35	1.60	67	26.2	54	TB	ZVD, DDI, T-SMZ
3	40	1.80	71	21.9	80	PCP	DDC, PA, BT
4	43	1.70	54	18.7	2	Encephalopathy	ZVD, T-SMZ, FC, DHPG, ZC, AC, PZ, FA
5	37	1.84	71	21.0	7	PCP	ZVD, T-SMZ
6	40	1.70	61	21.1	85	TB	ZVD, PA
7	36	1.76	64	20.7	295	KS	ZVD, DDI, T-SMZ, FD
Mean \pm SEM ¹	37 \pm 2	1.74 \pm 0.03	62 \pm 3	20.8 \pm 1.2	76 \pm 39		
Controls							
Mean \pm SEM	24 \pm 1	1.78 \pm 0.01	69 \pm 2	21.6 \pm 0.5			

¹ Values are means \pm SEM, n = 8.

Abbreviations: BMI, body mass index; FE, fungal esophagitis; VZ, herpes zoster; TB, lung tuberculosis; PCP, pneumocystis carinii pneumonia; KS, Kaposi sarcoma; DDI, Didanosine; ZVD, Zidovudine; T-SMZ, Sulfamethoxazole and Trimethoprim; DDC, Dideoxycytidine; PA, Pentamidine aerosol; BT, Betamethasone tablets; FC, Fluconazole; DHPG, Gancyclovir; ZC, Zopiclone; AC, Amoxicillin; PZ, Pericizine; FA, folic acid; FD, fusidic acid.

to define the amino acids that are most limiting for protein anabolism in AIDS patients on the basis of plasma free amino acid response (Pion 1973, Tontisirin et al. 1974, Zello et al. 1995) to a short-term intravenous infusion of an amino acid-glucose mixture.

SUBJECTS AND METHODS

Subjects. The study group consisted of seven men aged 30–43 (median 37 y) recruited from the Department of Infectious Diseases at the University Hospital in Clermont-Ferrand. On the basis of the ELISA and Western blot assay, all were HIV seropositive. A clinical history and a physical examination were performed at the time of the study (Table 1). Six patients were classified C3 (lymphocyte count <200) and one C2 (T4 lymphocytes between 200 and 499) according to the criteria of the Centers for Diseases Control and Prevention (1993). Only patients who had been free of any clinically active opportunistic infection for a period of ≥ 1 mo before participation were included. The patients' body weight loss was $\sim 5\%$ [compared with pre-illness body weight (BW)]. Patients with fever ($>37.8^\circ\text{C}$) or diarrhea (defined as increased frequency or liquidity of stools) were excluded. Eight male volunteers, who were HIV negative and clinically well, served as a control group. All patients gave a written informed consent. The study protocol was approved by the local Ethics Committee (Comité Consultatif pour la Protection des Personnes en Recherche Biomédicale pour la Région Auvergne).

Experimental procedure. All studies were performed in a postabsorptive state (12-h overnight fast). At 0800 h, a sampling catheter (Venflon 2, 20G, Viggo, Helsingborg, Sweden) was inserted into a dorsal vein of the left forearm. Another catheter was placed in a contralateral forearm vein and used for infusions. Each experiment consisted of a 150-min period of continuous infusion of the amino acid mixture Primene 5% (1 mL/(kg · h); Clintec Technologies, Vélizy-Villacoublay, France). Infusions were performed using a peristaltic pump (Infusomat Secura, Braun Biotrol, Paris). The nitrogen content of the amino acid mixture was 7.5 g/L and the amino acid concentration (g/L) was as follows: L-isoleucine 3.35, L-leucine 5.00, L-valine 3.80, L-lysine 5.50, L-methionine 1.20, L-phenylalanine 2.10, L-threonine 1.85, L-tryptophan 1.00, L-alanine 4.00, L-arginine 4.20, L-aspartic acid 3.00, L-cysteine hydrochloride 1.23, L-glutamic acid 5.00, glycine 2.00, L-histidine 1.90, L-proline 1.50, L-serine 2.00, L-tyrosine 0.45, L-ornithine 1.13 and taurine 0.30. A glucose solution (100 g/L; Meram, Melun, France) was concomitantly administered at a constant rate [1 mL/(kg · h)] via the same catheter by a separate

(–15 and –5 min), during (15, 30, 60, 90, 120 and 150 min) and after the infusion (15 and 30 min). Samples were collected in heparinized tubes, centrifuged at 4°C for 6 min at 3000 mg and plasma stored at -20°C for subsequent analysis.

Assays. Plasma tryptophan was determined by a fluorometric procedure after conversion to norharman by heating in acid conditions with formaldehyde and ferric chloride (Tesseraud et al. 1992). Plasma cyst(e)ine was determined directly by spectrophotometry after coloration with the acid ninhydrin reagent (Malloy et al. 1981). Plasma samples were prepared for analysis of other amino acids by mixing 3 mL plasma with 7 mL of 150 g/L trichloroacetic acid containing 0.16 mL thioglycol (to prevent methionine oxidation) and 0.75 μmol norleucine (as an internal standard). After storage at 0°C for 1 h and centrifugation at $4000 \times g$ for 30 min at 4°C , the supernatant was passed through a 3-mL cation-exchange column (Dowex AG 50WX8, 100–200 mesh; Bio-Rad, Richmond, CA). Amino acids were eluted from the column with 4 mol/L NH_4OH . The eluate was evaporated to dryness under reduced pressure at 50°C and reconstituted with 3 mL of 0.1 mol/L lithium buffer, pH 2.2. The concentrations of individual amino acids were determined by an automated ion-exchange chromatography apparatus (Biotronic LC 3000, Roucaire, Vélizy, France with BTC 2410 resin); utilizing postcolumn ninhydrin derivatization.

The concentration of other plasma substrates was determined by using an automatic enzymatic analyzer (Chem 1, Bayer Diagnostic, Puteaux, France) with the following enzymes: hexokinase and glucose-6-phosphate dehydrogenase for glucose; urease and glutamate dehydrogenase for urea; cholesterol esterase, cholesterol oxidase and peroxidase for cholesterol; and lipase, glycerokinase, pyruvate kinase and lactate dehydrogenase for triglycerides. Plasma insulin was determined with an immunoenzymatic assay kit (Abbott Diagnostics, Rungis, France). Prealbumin, albumin, retinol binding protein, α_1 acid-glycoprotein and C-reactive protein were measured by immunonephelometry (Beckman, Array System 360, Gagny, France).

Statistical analysis. All data are expressed as means \pm SEM. A paired *t* test was used to compare basal data with data obtained during infusions. A two-way ANOVA ($\alpha = 0.05$, main effects: HIV infection and glucose-amino acid infusion) was used to compare results in HIV-infected subjects and controls.

RESULTS

Plasma cholesterol, albumin, prealbumin, retinol binding protein, triglycerides, and C-reactive protein did not differ

TABLE 2

Routine biochemical variables of the acquired immunodeficiency syndrome (AIDS) patients and control subjects

	Controls ¹	AIDS ²
Cholesterol, mmol/L	4.13 ± 0.22	4.16 ± 0.49
Triglycerides, mmol/L	0.85 ± 0.09	1.76 ± 0.63
Prealbumin, g/L	0.32 ± 0.02	0.30 ± 0.03
Albumin, g/L	39.0 ± 1.1	34.9 ± 1.8
Retinol binding protein, mg/L	42.4 ± 1.9	45.7 ± 2.7
α ₁ -Acid glycoprotein, g/L	0.64 ± 0.06	0.92 ± 0.08*
C-reactive protein, mg/L	<1	<1

¹ Values are means ± SEM, *n* = 8.

² Values are means ± SEM, *n* = 7. **P* < 0.05 vs. controls.

between AIDS patients and controls (Table 2). The C-reactive protein was used as a marker to establish the lack of opportunistic infection at the time of the study. By contrast and as expected, the α₁ acid glycoprotein was greater (*P* < 0.05) in AIDS patients than in controls.

Basal plasma insulin concentrations (i.e., before the infusions) did not differ in HIV-infected subjects and in controls (Fig. 1). Plasma insulin was stable during the 90- to 50-min infusion period (the CV was 6.4 ± 3.4 and 7.6 ± 3.9% in controls and AIDS patients, respectively) and was not different between the two groups. Nevertheless, the infusion of amino acids plus glucose increased the plasma insulin concentration in AIDS patients (*P* < 0.05) but not in controls (Fig. 1).

Basal plasma glucose concentrations (Fig. 1) did not differ between HIV-infected subjects and controls. The concentrations increased (*P* < 0.05) in the two groups during the first 30-min period of infusions and thereafter reached a plateau (the mean cv was 4.7 ± 0.6 and 3.2 ± 0.4% for the controls and the AIDS patients, respectively, during the 30 to 150-min infusion period). The plateau concentration did not differ between nor did the increase in plasma glucose concentrations above basal. Plasma urea concentrations (Fig. 1) did not differ between groups and decreased significantly to the same extent (*P* < 0.05) during the amino acid-glucose infusion.

In the basal state (Table 3), the plasma concentrations of free methionine, threonine, histidine, isoleucine, leucine and tryptophan were significantly lower (*P* < 0.05) in AIDS patients than in controls. There was also a significantly lower concentration (*P* < 0.05) of the nonessential amino acids citrulline, glycine and aspartate plus asparagine in HIV patients. All other amino acid concentrations were not significantly different between groups.

The plasma concentrations of most essential free amino acids significantly increased (*P* < 0.05) during the amino acid-glucose infusion in both groups (Fig. 2). However, the absolute increase above basal levels (Fig. 3) was significantly lower (*P* < 0.05) in the HIV-infected subjects than in controls for threonine, valine and lysine. This also tended to be the case for methionine (*P* = 0.073). Amino acids increased mainly in the first 30 min of infusion and then reached a plateau (mean cv between 1.3 and 6.5% during 90–150 min). For all amino acids, the concentrations returned to the pre-amino acid infusion concentrations 30 min after termination of the infusions (Fig. 2). Phenylalanine concentration significantly increased (*P* < 0.05) during infusion in the controls but not in the HIV-infected subjects (Fig. 3). Tyrosine decreased (*P* < 0.05) similarly in both groups during infusion. A similar pattern was

seen for cyst(e)ine in controls but not in AIDS patients (*P* < 0.05 vs. controls).

Nonessential amino acid concentrations during glucose-amino acids infusions also differed for AIDS patients and control subjects (Fig. 4). For example, alanine greatly increased and aspartate plus asparagine decreased in controls (*P* < 0.05) but did not change in HIV-infected subjects. The absolute increase in glycine above basal was significantly lower (*P* < 0.05) in AIDS patients than in controls. All other nonessential amino acids were either similarly increased (serine, glutamine plus glutamate and ornithine; *P* < 0.05 vs. basal) or unchanged (citrulline and proline) during infusions in both groups.

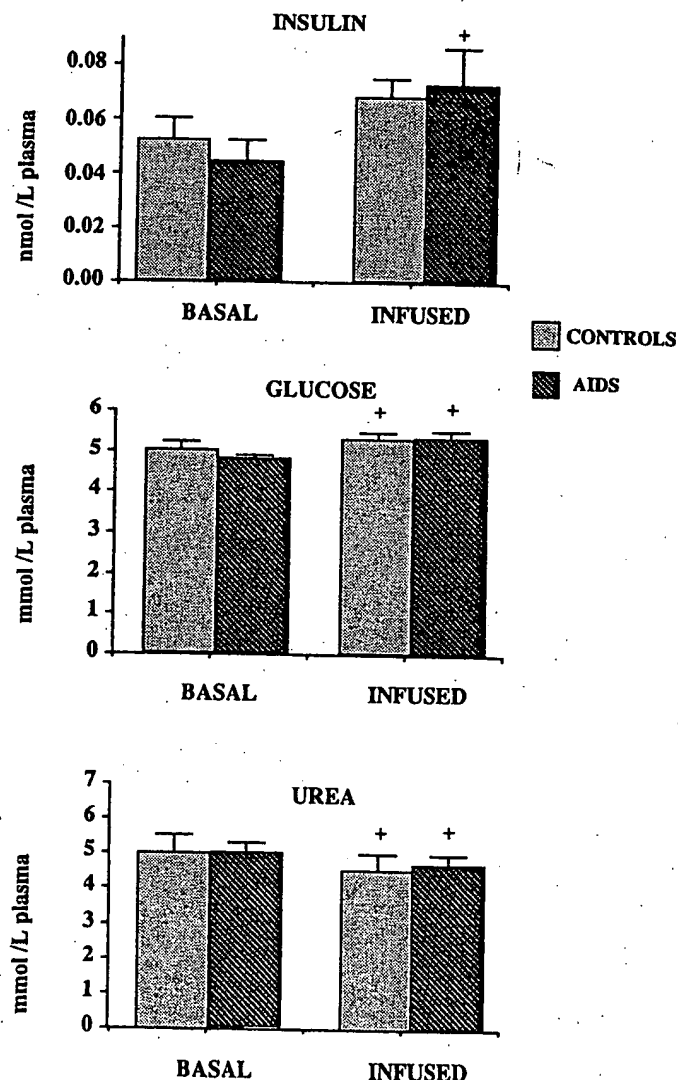


FIGURE 1 Plasma insulin, glucose and urea concentrations in patients with acquired immunodeficiency syndrome (AIDS) and control subjects. All variables were measured before (BASAL) and during the combined amino acid-glucose infusion (INFUSED). Values are means ± SEM for eight controls and seven AIDS patients. The insulin values represent one determination at time -15 min (before infusions, i.e., basal state) and the mean of three determinations at times 90, 120 and 150 min during infusions. The glucose and urea plasma values are the mean of two determinations in the basal state, i.e., times -15 and -5 min (before infusion), and five determinations at times 30, 60, 90, 120 and 150 min during infusions. *INFUSED significantly different from BASAL, *P* < 0.05.

TABLE 3

Basal plasma free amino acids in control subjects and acquired immunodeficiency syndrome (AIDS) patients

	Controls ¹	AIDS ²	Difference
	$\mu\text{mol/L}$		%
Essential			
Threonine	123 \pm 3	90 \pm 9*	-27
Valine	241 \pm 9	209 \pm 15	-13
Cyst(e)ine	192 \pm 8	179 \pm 7	-6
Methionine	22 \pm 1	16 \pm 2*	-30
Isoleucine	63 \pm 3	52 \pm 4*	-18
Leucine	122 \pm 4	101 \pm 9*	-17
Tyrosine	57 \pm 3	56 \pm 4	-2
Phenylalanine	56 \pm 1	59 \pm 5	+5
Lysine	163 \pm 4	163 \pm 13	0
Histidine	72 \pm 2	59 \pm 3*	-18
Arginine	75 \pm 3	66 \pm 6	-11
Tryptophan	59 \pm 2	51 \pm 3*	-14
Nonessential			
Aspartate + asparagine	97 \pm 3	78 \pm 4*	-19
Serine	108 \pm 7	95 \pm 9	-12
Glutamate + glutamine	422 \pm 30	389 \pm 54	-8
Glycine	226 \pm 10	183 \pm 11*	-19
Alanine	324 \pm 11	277 \pm 27	-14
Citrulline	32 \pm 1	22 \pm 2*	-31
Ornithine	44 \pm 1	48 \pm 2	+10
Proline	231 \pm 24	165 \pm 22	-28

¹ Values are means \pm SEM, $n = 8$.

² Values are means \pm SEM, $n = 7$. * $P < 0.05$ vs. controls.

DISCUSSION

Loss of lean body mass in HIV patients can result from undernutrition or from disease-induced alterations in metabolism. The lower basal plasma concentrations of most essential ($P < 0.05$ for threonine, methionine, isoleucine, leucine, histidine and tryptophan) and nonessential ($P < 0.05$ only for aspartate plus asparagine, glycine and citrulline) free amino acids in patients with AIDS are consistent with a state of protein undernutrition. Indeed, it has been found that reducing protein supply in rats during constant energy intake results in a decrease in the concentrations of most free amino acids in plasma (Grizard et al. 1977). However, the lower concentrations of plasma free amino acids in HIV patients could not be due to protein deprivation because dietary protein assessment (based on an 8-d diet recall) suggests a more than adequate daily protein intake (2.0 ± 0.3 g/kg BW). In addition, no specific energy restriction was detected (daily intake at 222 ± 29 kJ/kg BW) in these subjects. Our study therefore adds evidence to the hypothesis that a state of undernutrition does not exist in stable HIV patients, defined as those free of clinically active opportunistic infections, fever and diarrhea (Sauerwein 1993). However, these adequate intakes did not preclude muscle wasting, and many HIV patients have a history of long-term weight loss.

The lower concentrations of plasma free amino acids in fasting AIDS patients are presumably more a reflection of a stressed state associated with increased protein turnover and net catabolism. Most plasma free amino acid concentrations are decreased during stress, trauma and sepsis (Sax et al. 1988, Vente et al. 1989). Although HIV infection demonstrates many of the characteristics of a catabolic process, the initial

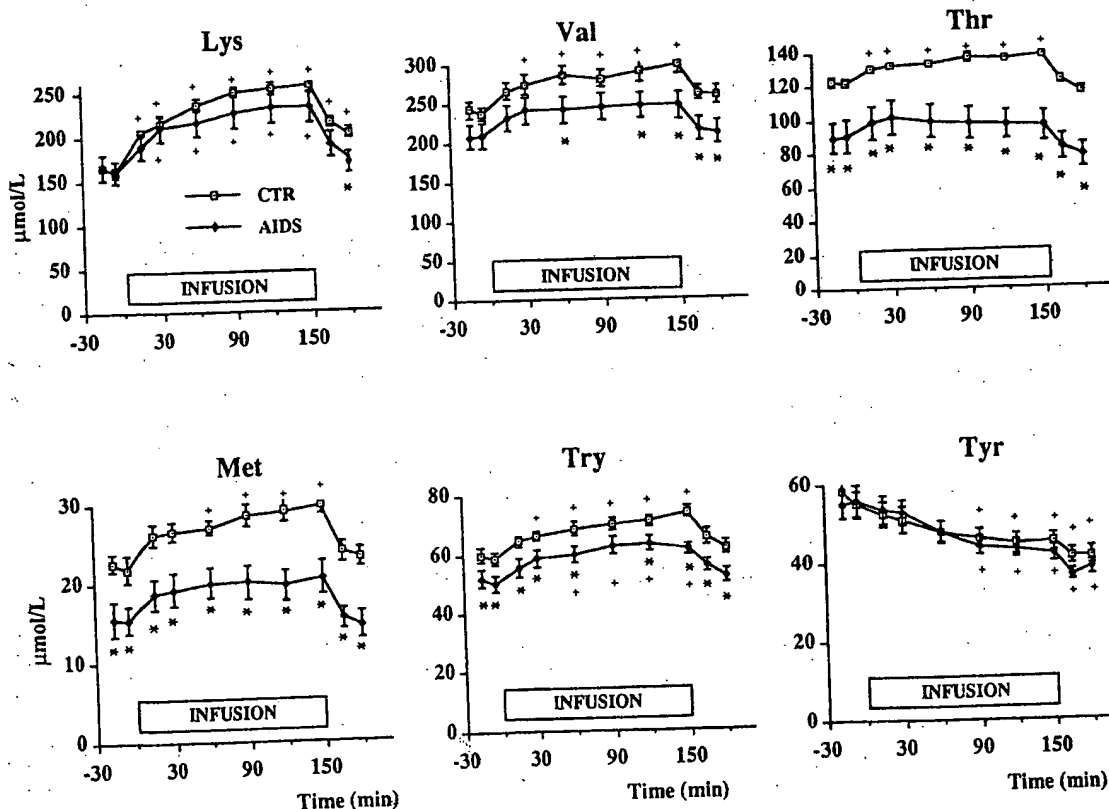


FIGURE 2 Plasma free amino acid concentrations in patients with acquired immunodeficiency syndrome (AIDS) and control subjects. Amino acids were assayed at various times in each group during a basal period (times -15 and -5 min), during the combined amino acid-glucose infusions (indicated by open boxes) and after the infusions (times 165 and 180 min). Values are means \pm SEM for eight controls and seven AIDS patients. *Significantly different from controls, $P < 0.05$. *Significantly different from the values at -15 or -5 min in the same group, $P < 0.05$.

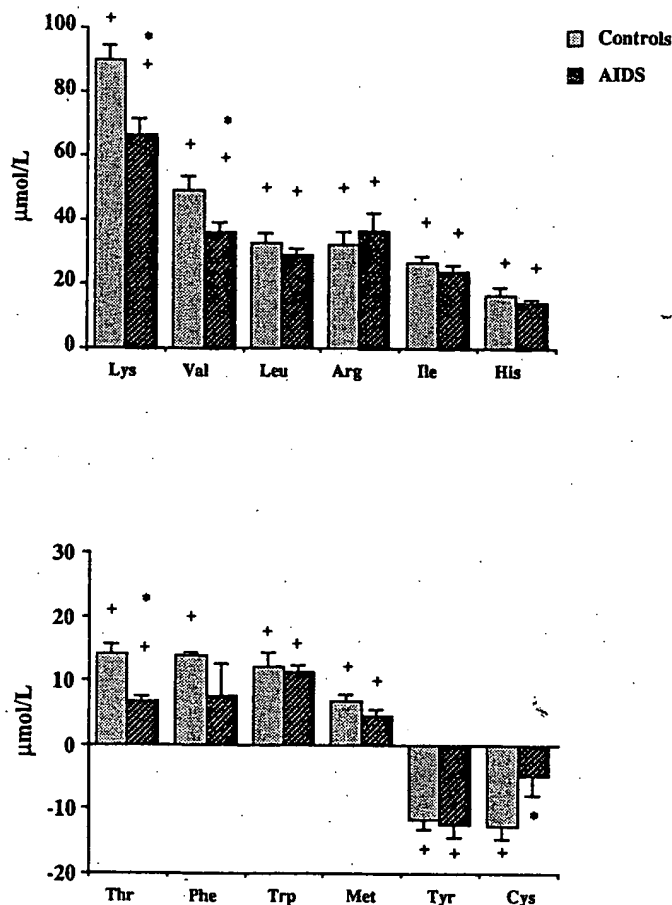


FIGURE 3 The absolute increase in plasma essential free amino acid concentrations during combined amino acid-glucose infusions in control subjects and acquired immunodeficiency syndrome (AIDS) patients. The plasma free amino acid concentrations were measured at times -15 and -5 min (before infusions) and at times 90, 120 and 150 min during infusions. The absolute increase of each amino acid represents the difference between the mean concentration obtained during and before infusions. Values are means \pm SEM for eight controls and seven human immunodeficiency virus (HIV)-infected patients. *Significantly different from controls, $P < 0.05$. *Significantly different from zero, $P < 0.05$.

study of protein metabolism in HIV infection using [^{15}N] glycine as a tracer (Stein et al. 1990) found reduced rates of whole-body protein turnover. However, more recent investigations using [^{13}C] leucine have suggested that the rates of protein turnover are high in cachectic AIDS patients (Lieberman et al. 1994; Macallan et al. 1995).

Increased protein turnover and catabolism should increase dietary protein requirements. Our study was therefore conducted to identify possible changes in individual amino acid requirements in patients with AIDS. Dietary amino acid requirements for adult humans have been determined by a number of different methods. Historically, descriptive or gross measurements such as nitrogen balance have been used. However, technological advancements have resulted in the use of more precise and mechanistic metabolic approaches to examine requirements (i.e., plasma amino acid concentrations, amino acid oxidation and indicator amino acid oxidation).

Our approach is based on the fact that when amino acids are provided at insufficient levels (limiting amino acids), most of these amino acids will be used efficiently for protein synthesis, and plasma free concentrations and oxidation will remain

low and constant. In contrast, the amino acids in excess of the amounts needed for protein synthesis accumulate and are preferentially oxidized by the body. As the supply of the limiting amino acids increases above the requirements for protein synthesis, increased concentration and catabolism of these amino acids ensue. This method, initially developed in animal experiments, has also been used in human studies (Fuller and Garlick 1994, McLarney et al. 1996, Zello et al. 1995).

On the basis of this concept, limiting amino acids should be able to be identified from plasma free amino acid concentrations in response to a fixed amino acid infusion. During amino acid infusion, the inhibition of protein synthesis and anabolism due to limiting amino acids will be suppressed; as a result, more amino acids will be incorporated into body proteins. The concentrations of nonlimiting amino acids will be altered, depending on the difference between supply by the perfusion and utilization for body deposition and oxidation. The plasma concentrations of limiting amino acids will stay at a low level if their supply from the infusion compensates only for their utilization. These amino acids will accumulate when in excess. In other words, amino acids that have a low basal level and do not change during infusion give indication that they are limiting amino acids for protein anabolism.

Studies investigating the fate of infused amino acids are consistent with these concepts. It has been shown in healthy volunteers that an infusion of mixed amino acids stimulates whole-body leucine disappearance through both oxidative and nonoxidative pathways (Bennet et al. 1989, Castellino et al. 1987 and 1992, Fukagawa et al. 1989, Pacy et al. 1988, Tessari et al. 1987). An even greater inhibition of endogenous leucine appearance was also seen when amino acids were combined with glucose and insulin (Bennet et al. 1990, Castellino et al. 1987, Flakoll et al. 1989, Fukagawa et al. 1989, Heslin et al. 1992, Tauveron et al. 1995, Tessari et al. 1987). The splanchnic bed is the major site of the disposal of intravenously administered amino acids (Gelfand et al. 1986). Studies in animals suggest that intravenous amino acids are catabolized preferentially by the liver and thus reduce the amounts of amino acids arising from proteolysis (Mortimore et al. 1987). Liver protein

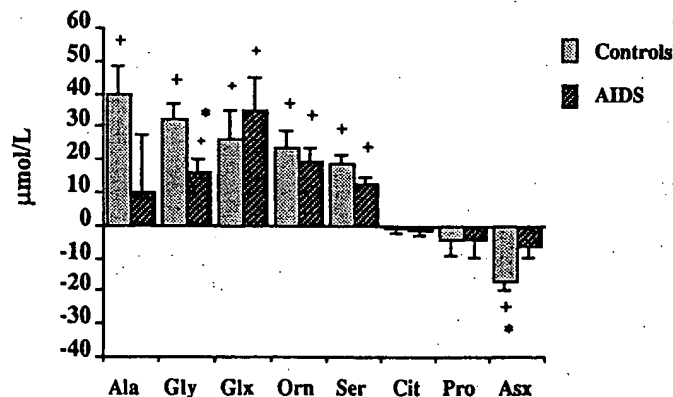


FIGURE 4 The absolute increase of plasma nonessential free amino acid concentrations during combined amino acid-glucose infusions in control subjects and acquired immunodeficiency syndrome (AIDS) patients. The plasma free amino acid concentrations were measured at times -15 and -5 min (before infusions) and at times 90, 120 and 150 min during infusions. The absolute increase of each amino acid represents the difference between the mean concentration obtained during and before infusions. Values are means \pm SEM for eight controls and seven AIDS patients. *Significantly different from controls, $P < 0.05$. *Significantly different from zero, $P < 0.05$. Glx = Glu + Gln. Asx = Asp + Asn.

synthesis may also be stimulated (Tauveron et al. 1994). Amino acid deposition in skeletal muscle cannot be ruled out because an increase in muscle protein synthesis by hyperaminoacidemia has been reported in both healthy volunteers (Bennet et al. 1989) and animals (Mosoni et al. 1993, Watt et al. 1992). Interestingly, it has been shown that the acute anabolic response to intravenous amino acid infusion was normal in HIV-infected subjects (Macallan et al. 1995, Selberg et al. 1995).

The magnitude of change in most essential plasma free amino acid concentrations during infusion in this study (an increase in leucine, arginine, isoleucine, histidine and tryptophan and a decrease in tyrosine) was similar in controls and HIV-infected subjects. Because the infusion rate was the same in the two groups, the changes in protein metabolism were roughly the same in the two groups. However, the increase in some other essential amino acids was smaller in HIV patients (lysine, valine and threonine) than in control subjects. This could be a reflection of increased basal protein turnover and amino acid oxidation.

Of the limiting amino acids, only the essential amino acids, threonine and perhaps methionine met our criteria (see above). For example, the basal level of threonine was among the most depressed of the amino acids in HIV-infected subjects (-27% compared with controls). In addition, the absolute increase in plasma free threonine after infusions (although significant, $P < 0.05$) was very modest in HIV-infected subjects, representing only 52% of the increase in controls. Based on the curve describing blood free threonine in response to the consumption of graded levels of threonine (Pion 1973, Tontisirin et al. 1974), our results suggest that HIV-infected patients have a selective deficiency in threonine. Such a deficiency has also been demonstrated recently in septic rats by using amino acid balance methodologies (Amal et al. 1995). This selective threonine deficiency could arise from an activation of the catabolism of threonine and/or synthesis of threonine-rich proteins.

A minor change in methionine was also noted after infusion, along with low basal levels in HIV-infected subjects. This amino acid deficiency is consistent with the known alterations in sulfur amino acid metabolism that occur in AIDS patients. An activation of the metabolism of cyst(e)ine, especially to taurine, may occur in HIV-infected patients (Hortin et al. 1994). To explain the decrease in plasma free methionine, we hypothesize that in patients with AIDS, there is a concomitant activation of the metabolism of methionine to cyst(e)ine. In contrast to previous studies (Hortin et al. 1994), cyst(e)ine depletion was not observed in our experiments although abnormal kinetics were recorded during infusions. We also hypothesize that more sulfur amino acids are needed in patients with AIDS to meet their requirements for glutathione (γ -glutamyl-cysteinylglycine) synthesis. Cysteine is both a precursor and a regulator of glutathione synthesis. HIV-infected patients are glutathione deficient (López Galera et al. 1996), presumably as a result of an enhanced utilization due to activation of lymphocytes and cell-mediated cytotoxic function and protection against oxidative damage. Similar mechanisms have been proposed as explanations for the increased sulfur amino acid requirements during sepsis in rats (Malmezat et al. 1998).

Previous studies indicated that the degradation of tryptophan via the kynurenine pathway is stimulated in HIV-infected subjects (Werner et al. 1988). This may contribute to the neurologic symptoms often associated with the HIV infection. Although the basal plasma tryptophan concentrations were significantly lower in HIV patients than in controls, this amino acid increased similarly after infusion in both groups

in our experiment and thus could not be considered rate limiting as was the case for threonine and methionine.

It is noteworthy that the nonessential amino acid glycine, exhibited the same behavior as a limiting amino acid. Glycine is metabolically related to serine, methionine, cysteine and threonine. These related amino acids are very abundant in many proteins synthesized in increased amounts during infection, trauma and chronic inflammatory diseases (see Grimble 1990 for a review). In this study, the utilization of glycine for glutathione synthesis was also presumably enhanced (see above). Alternatively, the appearance rate of glycine may have been decreased, first, because glycine synthesis from serine may have been decreased as a result of an increased utilization of serine for cysteine synthesis and second, because glycine can also be synthesized from threonine. We speculate that changes in threonine metabolism led to a decrease in glycine appearance. A drastic decrease in plasma free glycine has been observed in patients in response to multiple traumas (Grimble 1990).

The postabsorptive concentrations of free amino acids in plasma from HIV-infected subjects are more consistent with a septic situation rather than a state of protein deprivation or energy restriction. By using acute amino acid plus glucose infusions, we were able to detect selective amino acid deficiencies, especially with threonine and methionine. Methionine depletion correlated with the known alterations in sulfur amino acid metabolism during AIDS. In contrast, threonine depletion is a new concept that should be taken into account in AIDS nutrition. Further studies are needed to elucidate the changes in threonine metabolism and to determine whether this amino acid contributes to the pathophysiology of HIV infection.

ACKNOWLEDGMENTS

The authors thank Clintec Technologies for providing Primene 5% and Anthony Kee for improving the manuscript.

LITERATURE CITED

- Amal, M., Rosé, G., Breuillé, D. & Obled C. (1995) Composition à base d'acides aminés destinée au traitement du sepsis ou d'une agression engendrant une réaction inflammatoire chez les animaux et chez l'homme. Brevet n° 931288K publié en France.
- Bennet, W. M., Connacher, A. A., Scrimgeour, C. M., Jung, R. T. & Rennie, M. J. (1990) Euglycemic hyperinsulinemia augments amino acid uptake by human leg tissues during hyperaminoacidemia. *Am. J. Physiol.* 259: E185-E194.
- Bennet, W. M., Connacher, A. A., Scrimgeour, C. M., Smith, K. & Rennie, M. J. (1989) Increase in anterior tibialis muscle protein synthesis in healthy man during mixed amino acid infusion: studies of incorporation of [14 C] leucine. *Clin. Sci. (Lond.)* 76: 447-454.
- Boulétreau, P., Gérard, M., Messing, B., Chambrier, C., Gelas, P., Robert, D., Bryssine, S. & Khalallah, S. (1995) Home parenteral nutrition and AIDS. *Clin. Nutr.* 14: 213-218.
- Buhl, R., Jaffe, H. A., Holroyd, K. J., Wells, F. B., Mastrangeli, A., Saltini, C., Cantin, A. M. & Crystal, R. G. (1989) Systemic glutathione deficiency in symptom free HIV seropositive individuals. *Lancet* 2: 1294-1297.
- Castellino, P., Luzi, L., Simonson, D. C., Haymond, M. & DeFronzo, R. A. (1987) Effect of insulin and plasma amino acid concentrations on leucine metabolism in man. Role of substrate availability on estimates of whole body protein synthesis. *J. Clin. Invest.* 80: 1784-1793.
- Castellino, P., Solini, A., Luzi, L., Barr, J. G., Smith, D. J., Petrides, A., Giordano, M., Carroll, C. & DeFronzo, R. A. (1992) Glucose and amino acid metabolism in chronic renal failure—effect of insulin and amino acids. *Am. J. Physiol.* 262: F168-F176.
- Centers for Disease Control and Prevention (1993) Revised classification system for HIV infection and expanded surveillance case definition for AIDS among adolescents and adults. *Morb. Mortal. Wkly. Rep.* 41: 1-19.
- Chandra, R. K. (1991) Nutrition and immunity: lessons from the past and new insights into the future. *Am. J. Clin. Nutr.* 53: 1087-1101.
- Eck, H. P., Gmünder, H., Hartmann, M., Petzoldt, D., Daniel, V. & Dröge, W. (1989) Low concentrations of acid-soluble thiol (cysteine) in the blood plasma of HIV-1-infected patients. *Biol. Chem. Hoppe-Seyler* 370: 101-108.
- Flakoll, P. J., Kulaylat, M., Frexes-Steed, M., Hourani, H., Brown, L. L., Hill, J. O. &

- Abumrad, N. N. (1989) Amino acids augment insulin's suppression of whole body proteolysis. *Am. J. Physiol.* 257: E839-E847.
- Fukagawa, N. K., Minaker, K. L., Young, V. R., Matthews, D. E., Bier, D. M., & Rowe, J. W. (1989) Leucine metabolism in aging humans: effect of insulin and substrate availability. *Am. J. Physiol.* 256: E288-E294.
- Fuller, M. F. & Garlick, P. J. (1994) Human amino acid requirements: can the controversy be resolved? *Annu. Rev. Nutr.* 14: 217-241.
- Gelfand, R. A., Glickman, M. G., Jacob, R., Sherwing, R. S. & DeFronzo, R. A. (1986) Removal of infused amino acids by splanchnic and leg tissues in humans. *Am. J. Physiol.* 250: E407-E413.
- Gorbach, S. L., Knox, T. A. & Roubenoff, R. (1993) Interactions between nutrition and infection with human immunodeficiency virus. *Nutr. Rev.* 51: 226-234.
- Grimble, R. F. (1990) Nutrition and cytokine action. *Nutr. Res. Rev.* 3: 193-210.
- Grizard, J., Prugnaud, J. & Pion, R. (1977) Influence d'un excès d'insuline sur la composition corporelle et les teneurs en acides aminés libres du sang, du foie et du muscle du rat en croissance soumis ou non à une restriction azotée. *Ann. Biol. Anim. Biochim. Biophys.* 17: 373-387.
- Heslin, M. J., Newman, E., Wolf, R. F., Pisters, P. W. T. & Brennan, M. F. (1992) Effect of hyperinsulinemia on whole body and skeletal muscle leucine carbon kinetics in humans. *Am. J. Physiol.* 262: E911-E918.
- Hortin, G. L., Landt, M. & Powderly, W. G. (1994) Changes in plasma amino acid concentrations in response to HIV-1. *Clin. Chem.* 40: 785-789.
- Kotler, D. P., Wang, J. & Pierson, R. N. (1985) Body composition studies in patients with the acquired immunodeficiency syndrome. *Am. J. Clin. Nutr.* 42: 1255-1265.
- Lieberman, S. A., Butterfield, G. E., Harrison, D. & Hoffman, A. R. (1994) Anabolic effects of recombinant insulin-like growth factor-I in cachectic patients with the acquired immunodeficiency syndrome. *J. Clin. Endocrinol. Metab.* 78: 404-410.
- López Galera, R. M., Juárez Giménez, J. C., Montoro Ronsanó, J. B., Segura Cardona, R. M., Arbós Via, M. A., Altisent Roca, C. & Tusell Puigbert, J. M. (1996) Glutathione and cysteine in HIV-infected hemophiliacs. *Clin. Chim. Acta* 254: 63-72.
- Macallan, D. C., McNurlan, M. A., Milne, E., Calder, A. G., Garlick, P. J. & Griffin, G. E. (1995) Whole-body protein turnover from leucine kinetics and the response to nutrition in human immunodeficiency virus infection. *Am. J. Clin. Nutr.* 61: 818-826.
- Malloy, M. H., Rassin, D. K. & Gaul, G. E. (1981) A method for measurement of free and bound plasma cyst(e)ine. *Anal. Biochem.* 113: 407-415.
- Malmezzat, T., Breuillé, D., Pouyet, C., Patureau Mirand, P. & Obled, C. (1998) Metabolism of cysteine is modified during the acute phase of sepsis in rats. *J. Nutr.* 128: 97-105.
- McLarney, M. J., Pellett, P. L. & Young, V. R. (1996) Pattern of amino acid requirements in humans: an interspecies comparison using published amino acid requirements recommendations. *J. Nutr.* 126: 1871-1882.
- Melchior, J. C., Chastang, C., Gelas, P., Carbonnel, F., Zazzo, J. F., Boulter, A., Cosnes, J., Boulétreau, P. & Messing, B. (1996) Efficacy of 2-month total parenteral nutrition in AIDS patients: a controlled randomized prospective trial. *AIDS* 10: 379-384.
- Mortimore, G. E., Pösö, A. R., Kadowaki, M. & Wert, J. J. (1987) Multiphasic control of hepatic protein degradation by regulatory amino acids. General features and hormonal modulation. *J. Biol. Chem.* 262: 16322-16327.
- Mosoni, L., Houlier, M. L., Patureau Mirand, P., Bayle, G. & Grizard, J. (1993) Effect of amino acids alone or with insulin on muscle and liver protein synthesis in adult and old rats. *Am. J. Physiol.* 264: E614-E620.
- Pacy, P. J., Garrow, J. S., Ford, G. C., Merritt, H. & Halliday, D. (1988) Influence of amino acid administration on whole-body leucine kinetics and resting metabolic rate in postabsorptive normal subjects. *Clin. Sci. (Lond.)* 75: 225-231.
- Pellet, P. L. (1990) Protein requirements in humans. *Am. J. Clin. Nutr.* 51: 723-737.
- Pion, R. (1973) The relationships between the levels of free amino acids in blood and muscle and the nutritive value of proteins. In: *Protein in Human Nutrition* (Porter, J. W. & Rolfs, B. A., eds.), pp. 329-341. Academic Press, London, UK.
- Reeds, P. J., Fjeld, C. R. & Jahoor, F. (1994) Do the differences between the amino acid compositions of acute-phase and muscle proteins have a bearing on nitrogen loss in traumatic states? *J. Nutr.* 124: 906-910.
- Sauerwein, H. P. (1993) Nutrition and AIDS. *Clin. Nutr.* 12: S64-S68.
- Sax, H. C., Hasselgren, P.-O., Talamini, M. A., Edwards, L. L. & Fischer, J. E. (1988) Amino acid uptake in isolated, perfused liver: effect of trauma and sepsis. *J. Surg. Res.* 45: 50-55.
- Selberg, O., Söttmann, U., Melzer, A., Deicher, H., Müller, M. J., Henkel, E. & McMillan, D. C. (1995) Effect of increased protein intake and nutritional status on whole-body protein metabolism of AIDS patients with weight loss. *Metabolism* 44: 1159-1165.
- Stein, T. P., Nutinsky, C., Condoluci, D., Schluter, M. D. & Leskiw, M. J. (1990) Protein and energy substrate metabolism in AIDS patients. *Metabolism* 39: 876-881.
- Sukkar, S. G. & Giacosa, A. (1995) Home nutritional support in AIDS patients. *Clin. Nutr.* 14: 41-45.
- Tauveron, I., Charrier, S., Champredon, C., Bonnet, Y., Berry, C., Bayle, G., Prugnaud, J., Obled, C., Grizard, J. & Thiéblot, P. (1995) Response of leucine metabolism to hyperinsulinemia under amino acid replacement in experimental hyperthyroidism. *Am. J. Physiol.* 269: E499-E507.
- Tauveron, I., Larbaud, D., Champredon, C., Debras, E., Tesseraud, S., Bayle, G., Bonnet, Y., Thiéblot, P. & Grizard, J. (1994) Effect of hyperinsulinemia and hyperaminoacidemia on muscle and liver protein synthesis in lactating goats. *Am. J. Physiol.* 267: E877-E885.
- Tessari, P., Inchiostro, S., Biolo, G., Trevisan, R., Fantin, G., Marescotti, M. C., Iori, E., Tiengo, A. & Crepaldi, G. (1987) Differential effects of hyperinsulinemia and hyperaminoacidemia on leucine-carbon metabolism in vivo. *J. Clin. Invest.* 79: 1062-1069.
- Tesseraud, S., Grizard, J., Makarski, B., Debras, E., Bayle, G. & Champredon, C. (1992) Effect of insulin in conjunction with glucose, amino acids and potassium on net metabolism of glucose and amino acids in the goat mammary gland. *J. Dairy Res.* 59: 135-149.
- Tontisirin, K., Young, V. R., Rand, W. M. & Scrimshaw, N. S. (1974) Plasma threonine response curve and threonine requirements in young men and elderly women. *J. Nutr.* 104: 495-505.
- Vente, J. P., Von Meyenfeldt, M. F., Van Eijk, H. M. H., Van Berlo, C. L. H., Gouma, D. J., Van der Linden, C. J. & Soeters, P. B. (1989) Plasma-amino acid profiles in sepsis and stress. *Ann. Surg.* 209: 57-62.
- Watt, P. W., Corbett, M. E. & Rennie, M. J. (1992) Stimulation of protein synthesis in pig skeletal muscle by infusion of amino acids during constant insulin availability. *Am. J. Physiol.* 263: E453-E460.
- Werner, E. R., Fuchs, D., Hausen, A., Jaeger, H., Reibnegger, G., Werner-Felmayer, G., Dierich, M. P. & Wachter, H. (1988) Tryptophan degradation in patients infected by human immunodeficiency virus. *Biol. Chem. Hoppe-Seyler* 369: 337-340.
- Zello, G. A., Wykes, L. J., Ball, R. O. & Pencharz, P. B. (1995) Recent advances in methods of assessing dietary amino acid requirements for adult humans. *J. Nutr.* 125: 2907-2915.

Niacin as a potential AIDS preventive factor

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Summary A pentad of findings consistent with niacin depletion have been described in patients with AIDS. There are also clinical and laboratory data to support the potential benefit of niacin in HIV infection. In this paper, it is hypothesized that HIV infection induces niacin depletion, and that therapeutic niacin will act as an AIDS preventive factor. While viral inhibition is incontrovertibly the primary 'AIDS preventive factor', costly antiretroviral medications are simply out of reach for the majority of the world's HIV-infected people. Along with antiviral research, investigation must go forward to look at strategies to overcome the massive metabolic disruption caused by the production of approximately one billion virus particles per day. Niacin, the same B complex vitamin found in the early part of this century to be the 'pellagra preventive factor', is proposed here as a secondary 'AIDS preventive factor' in HIV-infected persons. © 1999 Harcourt Publishers Ltd

INTRODUCTION

This hypothesis is built on three related concepts: (1) niacin depletion in the absence of dietary deficiency; (2) infection-induced vitamin depletion; and (3) improved clinical outcome associated with therapeutic use of vitamins during infection. All three of these concepts have proofs of principle independent of the HIV pandemic. Carcinoid syndrome can lead to niacin depletion in the absence of dietary deficiency (1). In carcinoid syndrome, the body's tryptophan is diverted by tumor metabolism from niacin production towards serotonin production, this shunting leading to pellagra in some carcinoid patients (2). Infection-associated vitamin depletion and beneficial therapeutic vitamin use during infection have both been demonstrated with vitamin A during measles infection (3). The natural history of HIV infection is a state of progressive immune dysfunction occurring over a number of years and culminating in the clinical state of acquired immune deficiency syndrome (AIDS) (4). This syndrome has a number of poorly understood phenomena associated with it which have not been explained by direct viral effects and are hypothesized here to be a result of HIV-induced niacin depletion. If

therapeutic niacin finds a place as an AIDS preventive factor, this might then be a starting point for hope of affordable interventions in the developing world (5).

NIACIN

Niacin was identified as the pellagra preventive factor through the work of Joseph Goldberger and others (6). Despite prevailing theories of an infectious etiology, vitamin deficiency was ultimately proven to be the causative problem in epidemic pellagra (7). Since the 1930s, the biochemical basis of this problem has been clarified, and the list of etiologies has expanded beyond dietary deficiency to include drug-induced pellagra, pellagra induced by inborn metabolic errors, and disease-associated pellagra (8).

Niacin, or vitamin B3, is the accepted name for two related vitamin compounds: nicotinic acid [NA] and nicotinamide [NAM]. Niacin's metabolic fate is the synthesis of nicotinamide nucleotide compounds such as nicotinamide adenine dinucleotide [NAD] (9). NAM, unlike NA, can be cleaved from and then recycled back to nicotinamide nucleotides in vivo through the action of NAD hydrolases (9).

There are four biosynthetic pathways for NAD. Two of the pathways utilize NAM as the initial precursor, one starts with NA, and the final synthetic pathway starts with tryptophan. These biosynthetic pathways are each dependent on a series of enzymatic steps, and there is significant variability in the tissue distribution of the

Received 26 April 1999

Accepted 4 May 1999

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enzymes. The pathway with the widest tissue distribution is one of the two NAM-associated pathways (9).

TRYPTOPHAN

The essential amino acid tryptophan is critical to the understanding of pellagra. Dietary tryptophan's metabolic fate is threefold: (1) protein synthesis; (2) serotonin synthesis; (3) oxidative metabolism (10). In normal individuals, the body uses only 5% of tryptophan for the serotonin and protein synthesis pathways, and disposes of the remaining tryptophan along the kynurenine pathway, where the end products are either nicotinamide nucleotides or acetylCoA (10). The considerable volume of data on increased quinolinic acid in HIV infection suggests that increased conversion to nicotinamide nucleotides is occurring in HIV infection (11). Normally, approximately 1 in every 60 tryptophan molecules is converted to niacin (12). This understanding has led to the use of the term 'niacin equivalents[NES]' to describe total dietary intake of niacin as the sum of both vitamin intake and tryptophan-related synthesis.

PELLAGRA: A PENTAD

The three Ds of dermatitis, diarrhea, and dementia clinically identify pellagra. This handy mnemonic is confounded by clinical data which suggest that in pellagrins the full clinical triad is only present in a minority of cases (13). Spivak and Jackson found that only 22% of pellagrins in their series had the full triad. Even dermatitis, often anticipated as the *sine qua non*, was absent in 15% of historical cases reviewed (13).

The biochemical findings associated with pellagra have been known for some time (8). Although direct measures of serum niacin are possible, the work of Fu et al. suggests that the serum tryptophan and intracellular NAD are reliable measures of niacin depletion. In Fu's study, moderate niacin deficiency led to intracellular NAD decreases, while more severe niacin deficiency led to both intracellular NAD and serum tryptophan decreases (14). None of the subjects in Fu's study was reported to have developed clinical signs of dermatitis, diarrhea, or dementia during a five-week period of dietary niacin deficiency, suggesting that biochemical changes are a more sensitive monitor than clinical signs. Pellagra is marked by a total of five findings: dermatitis, diarrhea, dementia, decreased cellular NAD, and decreased serum tryptophan.

AIDS AND THE 3 Ds

Amongst a number of poorly understood clinical findings in AIDS patients are 'seborrheic' dermatitis, AIDS

enteropathy, and AIDS dementia complex (ADC). The dermatitis is usually referred to as 'seborrheic' on the basis of clinical appearance, but the histology in AIDS is felt to be atypical (15). AIDS enteropathy is viewed as a diagnosis of exclusion in seropositive patients with chronic diarrhea, who have no infectious cause determined after complete evaluation (16). Mucosal histopathology is remarkable for low-grade atrophy and a maturational defect in the enterocytes (17). ADC is marked pathologically by neuronal loss (18), and microglial nodules (19). With all three of these problems, scientists and clinicians have tried with only limited success to link the changes in the skin, mucosa, and the brain with localized presence of the virus itself. Recent reports associating the attenuation of two of these conditions with the use of highly active antiretroviral therapy[HAART] (20,21) suggest that the three Ds may be metabolic effects of viral production.

NAD AND TRYPTOPHAN IN HIV INFECTION

Cellular NAD depletion occurs in HIV-infected patients in T4 lymphocytes, T8 lymphocytes, and non-T lymphocytes (22). This depletion occurs in a progressive manner with advancing HIV disease. Lymphocytes from symptomatic HIV-infected patients have an impaired ability to produce NAD when stimulated (23). Along with these clinical observations are two *in vitro* observations that demonstrate that: (1) acute infection with HIV leads to a rapid decrease in NAD concentration (24); and (2) chronic infection with HIV leads to chronic depression of NAD concentration (25).

Numerous groups have observed decreased serum tryptophan levels in patients with HIV infection (26,27). It has also been observed that there are increased levels of intermediates of tryptophan oxidative metabolism along the NAD biosynthetic pathway (28). Recently, the observed depression of tryptophan levels have been shown to rebound with antiviral therapy (29).

These data, when taken together, suggest a metabolic shunting of tryptophan towards NAD synthesis in response to a virus-induced NAD depletion. If Fu's data apply, then the presence of both intracellular NAD depletion and serum tryptophan depletion suggests significant niacin depletion (14).

EVIDENCE FOR BENEFITS OF NIACIN IN HIV INFECTION

In vitro data demonstrate that niacin in the form of NAM acts as an inhibitor of HIV infection (30). This inhibition occurs in both acute and chronic infection models at millimolar concentrations. The inhibition takes place, at least in part, at a post-integrational step in the viral life-

cycle (30). It was also noted that this same compound, NAM, leads to an increase in intracellular NAD when added to infected cultures, a response that is otherwise impaired in HIV-infected cells (23,24).

There are no published data on the prospective use of therapeutic niacin to inhibit HIV *in vivo*. There have been two prospective observational studies, however, where increased dietary niacin was associated with improved clinical outcome (31,32). This correlation was statistically significant in the study by Tang and colleagues, who found that niacin intake exceeding 64 mg per day coincided with decreased risk of HIV disease progression and improved survival (32). This amount of niacin is only two to four times what the average diet provides (12). It is important to note that Tang's study also found survival benefit with some other vitamin compounds including vitamin B6. Pertinent to this discussion is the fact that vitamin B6 is a necessary cofactor in the conversion of tryptophan to NAD (33).

NIACIN DEPLETION: A QUESTION OF SUPPLY AND DEMAND

A mechanism for niacin depletion in HIV infection is not clear at this time. It is apparent that, in order of HIV to induce niacin depletion, there must be either a change in supply, demand or both (supply and demand) in the HIV-infected state. Since there are no stored niacin reserves in the body, niacin requirements are met by ingestion of the vitamin and the *de novo* synthesis from dietary tryptophan (13). Examination of dietary intake in HIV-infected individuals has failed to suggest specific deficiency in either NE or tryptophan (34). As discussed, there is a body of evidence which suggests that tryptophan's conversion to nicotinamide nucleotides is activated in the HIV-infected state (11,28). This increase in tryptophan oxidation makes undersupply of niacin an unlikely culprit in niacin depletion, unless there is a heretofore unidentified block in the pathway which converts tryptophan to NAD. Any suggestion of simply increasing dietary tryptophan should be viewed with caution given the potential for side-effects (35).

Overutilization of niacin seems more likely than undersupply, given the observation that HIV infection induces NAD depletion (23). *In vitro* data suggest that this is a direct effect of HIV on cellular NAD levels (24,25). The depletion of NAD, multiplied by the more than one billion T lymphocytes turned over per day during HIV infection and stretched over a period of several years could readily be expected to cause niacin depletion (4). NAD, the biologic end product of niacin, is depleted on an intracellular level by HIV and this implies that systemic niacin depletion is primarily tied to niacin overutilization in HIV-infected patients.

CD38: A POTENTIAL CLUE TO NIACIN DEPLETION

CD38 is a NAD hydrolase on the cell surface which catalyzes the conversion of extracellular NAD to NAM and ADP-ribose (36). Both CD4 and CD8 lymphocytes demonstrate an increased percentage of cells positive for CD38 with HIV seroconversion (37). CD38 numbers keep increasing in T lymphocytes with advancing HIV disease (38). Mehta et al. suggested that the changes in CD38+ population during disease progression imply that CD38 is endowed with 'peculiar protective function' (36). CD38 allows extracellular NAD, which can not enter intact cells, to be cleaved on the surface, thereby increasing NAM in the extracellular microenvironment. This NAM can then enter the cell and form NAD (39). CD38's ectoenzyme activity and the resultant increase in extracellular niacin are the likely explanations for the finding by Skurnik et al. that serum niacin levels increase with HIV infection (40). CD38 activity may also contribute to the T8 lymphocyte's antiviral activity since the NAM released has potential antiviral activity (30,41).

The importance of CD38 in HIV infection is becoming increasingly appreciated. CD38 was shown to be a stronger predictor of AIDS and death in HIV infection than CD4 counts in one study (42). CD38 numbers decrease with HAART, suggesting that control of HIV infection leads to diminished signaling for CD38 upregulation (43). We need to understand the role of CD38 in HIV infection more completely before the details of HIV-induced niacin metabolism can be fully understood.

CONCLUSION

This hypothesis, if supported by further study, may provide some hope to the 90% of the world's HIV-infected population who live beyond the reach of costly antiretroviral therapies (5). Niacin may also find a place as an adjunct to HAART. Clinical trials of niacin as an AIDS preventive factor are warranted by the following:

1. the clinical observation that a modest increase in dietary niacin is coincident with decreased risk for progression to AIDS or death in patients with HIV;
2. *in vitro* data showing niacin to be an inhibitor of HIV production;
3. the pentad of poorly explained phenomena in AIDS patients which coincides with the five characteristic findings in niacin depletion, i.e. (a) dermatitis; (b) diarrhea; (c) dementia; (d) intracellular NAD depletion; (e) serum tryptophan depletion;
4. the safety profile of niacin as a drug (44).
5. the wide availability of inexpensive niacin.

It will likely require a large trial group over a prolonged period in order to prove niacin therapy to be a useful

AIDS preventive factor. The chance of demonstrating this effect in Europe or the USA, where expensive antivirals have become the standard of care, may be difficult but, in the developing world, where scarce resources cry out for inexpensive therapeutic compounds, the time may be right for clinical trials of therapeutic niacin. Meanwhile, further study is necessary to delineate the metabolic details of HIV-induced niacin depletion.

REFERENCES

- Castiello R. J., Lynch P. J. Pellagra and the carcinoid syndrome. *Arch Derm* 1972; **105**: 574-577.
- Waldenstrom J., Ljungberg E. Studies on the functional circulatory influence from metastasizing carcinoid tumours and their possible relation to enteramine production. *Acta Med Scand* 1955; **152**: 293-309.
- Hussey G. D., Klein M. Measles induced vitamin A deficiency. *Ann NY Acad Sci* 1992; **669**: 188-194.
- Ho D. D. Dynamics of HIV-1 replication in vivo. *J Clin Invest* 1997; **99**: 2565-2567.
- Piot P. The science of AIDS: a tale of two worlds. *Science* 1998; **280**: 1844-1845.
- Bollet A. J. Politics and pellagra: the epidemic of pellagra in the US in the early twentieth century. *Yale J Biol Med* 1992; **65**: 211-221.
- Elvehjem C. A., Madden R. J., Strong F. M., Woolley D. W. Relation of nicotinic acid and nicotinic acid amide to canine black tongue. *J Am Chem Soc* 1937; **59**: 1767-1768.
- Truswell A. S., Hansen J. D. L., Wannenburg P. Plasma tryptophan and other amino acids in pellagra. *Am J Clin Nutr* 1968; **21**: 1314-1320.
- Shibata K., Hayakawa T., Taguchi H., Iwai K. Regulation of pyridine nucleotide coenzyme metabolism. In: Schwarcz R., ed. *Kyurenine and Serotonin Pathways*. New York: Plenum Press, 1991.
- Peters J. C. Tryptophan nutrition and metabolism: an overview. In: Schwarcz R., ed. *Kyurenine and Serotonin Pathways*. New York: Plenum Press, 1991.
- Heyes M. P. Metabolism and neuropathologic significance of quinolinic acid and kynurenic acid. *Biochem Soc Trans* 1993; **21**: 83-89.
- Henderson L. M. Niacin. *Ann Rev Nutr* 1983; **3**: 289-307.
- Spivak J. L., Jackson D. L. Pellagra: an analysis of 18 patients and a review of the literature. *Johns Hopkins Med J* 1977; **140**: 295-309.
- Fu C. S., Swendseid M. E., Jacob R. A., McKee R. W. Biochemical markers for assessment of niacin status in young men: levels of erythrocyte niacin coenzymes and plasma tryptophan. *J Nutr* 1989; **19**: 1949-1955.
- Soeprono F. F., Schinella R. A., Cockerell C. J. et al. Seborrheic-like dermatitis of acquired immunodeficiency syndrome. *J Am Acad Dermatol* 1985; **14**: 242-248.
- Greenston J. K., Belitsos P. C., Yardley J. H., Bartlett J. G. AIDS enteropathy: occult enteric infections and duodenal alterations in chronic diarrhea. *Ann Intern Med* 1991; **114**: 366-372.
- Ullrich R., Zeitz M., Heise W., L'age M., Hoffken G., Riecken E. O. Small intestinal structure and function in patients infected with HIV: evidence for HIV-induced enteropathy. *Ann Intern Med* 1989; **111**: 15-21.
- Everall I. P., Luthert P. J., Lantos P. L. Neuronal loss in the frontal cortex of HIV infection. *Lancet* 1991; **337**: 1119-1121.
- DeGirolami U., Smith T. W., Henin D., Hauw J. J. Neuropathology of the acquired immunodeficiency syndrome. *Arch Pathol Lab Med* 1990; **114**: 643-655.
- Foudraine N. A., Weverling G. J., VanGool T. et al. Improvement of chronic diarrhoea in patients with advanced HIV-1 infection during potent antiretroviral therapy. *AIDS* 1998; **12**: 35-41.
- Skolnick A. A. Protease inhibitors may reverse AIDS dementia. *JAMA* 1998; **279**: 419.
- Tabucchi A., Carlucci F., Consolmagno E. et al. Changes in purine nucleotide content in the lymphocyte subpopulations of patients infected with HIV. *Clinica Chimica Acta* 1994; **225**: 147-153.
- Bofill M., Fairbanks L. D., Ruckemann K., Lipman M., Simmonds H. A. T-lymphocytes from AIDS patients are unable to synthesize ribonucleotides de novo in response to mitogenic stimulation. *J Biol Chem* 1995; **270**: 29690-29697.
- Murray M. F., Nghiem M., Srinivasan A. HIV infection decreases intracellular nicotinamide adenine dinucleotide. *Biochem Biophys Res Commun* 1995; **212**: 126-131.
- Carlucci F., Tabucchi A., Rosi F. et al. Purine ribonucleotide content in infected HIV-RT+ and HIV-RT- lymphoblastoid cell lines. *Biomed Pharmacother* 1996; **50**: 158-162.
- Hortin G. L., Landt M., Powderly W. G. Changes in plasma amino acid concentrations in response to HIV-1 infection. *Clin Chem* 1994; **40**: 785-789.
- Fuchs D., Moller A. A., Reinegger G. et al. Increased endogenous interferon-gamma and neopterin coelate with increased degradation of tryptophan in human immunodeficiency virus type 1 infection. *Immun Lett* 1991; **28**: 207-212.
- Sardar A. M., Bell J. E., Reynolds G. P. Increased concentrations of the neurotoxin 3-hydroxykynurenine in the frontal cortex of HIV-1 positive patients. *J Neurochem* 1995; **64**: 932-935.
- Gisslen M., Larsson M., Norkrans G., Fuchs D., Wachter H., Hagber L. Tryptophan concentrations increase in cerebrospinal fluid and blood after zidovudine treatment in patients with HIV type 1 infection. *AIDS Res Hum Retrovir* 1994; **10**: 947-951.
- Murray M. F., Srinivasan A. Nicotinamide inhibits HIV-1 in both acute and chronic in vitro infection. *Biochem Biophys Res Commun* 1995; **210**: 954-959.
- Abrams B., Duncan D., Hertz-Picciotto I. A prospective study of dietary intake and acquired immune deficiency in HIV-seropositive homosexual men. *J AIDS* 1993; **6**: 949-958.
- Tang A. M., Graham N. M. H., Saah A. J. Effects of micronutrient intake on survival in human immunodeficiency type 1 infection. *Am J Epidemiol* 1996; **143**: 1244-1256.
- Ubbink J. B., Vermaak W. J. H., Bissobort S. H. High performance liquid chromatographic assay of human lymphocyte kynureninase activity levels. *J Chromat* 1991; **566**: 369-375.
- Sharkey S. J., Sharkey K. A., Sutherland J. R. et al. Nutritional status and food intake in human immunodeficiency virus infection. *J AIDS* 1992; **5**: 1091-1098.
- Centers for Disease Control. Eosinophilia myalgia syndrome and L-tryptophan containing products - New Mexico, Minnesota, Oregon, and New York. *MMWR* 1989; **38**: 785-788.
- Mehta K., Shahid U., Malavasi F. Human CD38, a cell-surface protein with multiple functions. *FASEB J* 1996; **10**: 1408-1417.
- Benito J. M., Zabay J. M., Gil J. et al. Quantitative alterations of the functionally distinct subsets of CD4 and CD8 T lymphocytes in asymptomatic HIV infection. *J AIDS HR* 1997; **14**: 128-135.
- Puppo F., Brenci S., Bosco O. et al. Downregulation of HLA class I antigen expression in CD4+ T lymphocytes from HIV type 1-infected individuals. *AIDS Res Hum Retrovir* 1997; **13**: 1509-1516.

39. Deterre P., Gelman L., Gary-Gouy H. et al. Coordinated regulation in human T cells of nucleotide-hydrolyzing ectoenzymatic activities including CD38 and PC-1. *J Immunol* 1996; **157**: 1381-1388.
40. Skurnick J. H., Bogden J. D., Baker H. et al. Micronutrient profiles in HIV-1-infected heterosexual adults. *J AIDS Hum Retrovirol* 1996; **12**: 75-83.
41. Levy J. A., Mackewicz C. E., Barker E. Controlling HIV pathogenesis: the role of the noncytotoxic anti-HIV response of CD8+ T cells. *Immunol Today* 1996; **17**: 217-224.
42. Liu Z., Cumberland W. G., Hultin L. E., Prince H. E., Detels R., Giorgi J. V. Elevated CD38 antigen expression on CD8+ T cells is a stronger marker for the risk of chronic HIV progression to AIDS and death in the Multicenter AIDS cohort study than CD4+ cell count, soluble immune activation markers, or combinations of HLA-DR and CD38 expression. *J AIDS HR* 1997; **16**: 83-92.
43. Silvestri G., Munoz C., Butini L., Bagnarelli P., Montroni M. Changes in CD8 cell subpopulations induced by antiretroviral therapy in human immunodeficiency virus infected patients. *Viral Immunol* 1997; **10**: 207-212.
44. DiPalma J. R., Thayer W. S. Use of niacin as a drug. *Annu Rev Nutr* 1991; **11**: 169-187.

THE LANCET

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Please quote ref: KAM/Dtc - 98/8358

30 September 1998

Dr MF Murray
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USA

Dear Dr Murray

Many thanks for submitting your hypothesis on niacin and AIDS to The Lancet.

We discussed your paper at an editorial meeting in light of the opinions of our peer reviewers. As you will see, our referees had some major concerns. Decisions about the suitability of a paper are made by editorial consensus, taking into consideration the interest to our readership, and the quality and suitability of the many other papers we consider each week. I am sorry to inform you that your paper did not meet our threshold for publication.

I wish you the best of luck for your paper elsewhere. Thank you for thinking of The Lancet.

Yours sincerely

A handwritten signature in dark ink, appearing to be "Dr Kelly Morris", written over the typed name.

Dr Kelly Morris
Senior Editor

EXHIBIT D

THE LANCET
MANUSCRIPT REVIEW

Manuscript number: 38/ 8350

Authors: Murray MF

Reviewer number: 4241

Please give a frank account of the strengths and weaknesses of the article.

COMMENTS FOR AUTHORS:

The author proposes that niacin may act as an "AIDS preventive factor". By this he means that it will prevent progression from asymptomatic infection to symptomatic disease. It is however likely to be misconstrued as a factor which may prevent infection with HIV virus. The author should however be commended in trying to suggest an inexpensive treatment which may be used world-wide rather than just in the Western world.

Comments

- Nicotinamide acts as an inhibitor of HIV infection at millimolar concentrations. What are the concentrations of NAM achieved in vivo? For the hypothesis to be tenable the concentrations achieved in vivo should be equivalent to those required to inhibit HIV infection in vitro.
- HIV infected patients have been shown to have elevated niacin levels but decreased serum tryptophan and NAD levels. The latter are said to be reliable markers of niacin deficiency, but this is derived from studies carried out in young non-HIV infected men. With all the different metabolic changes occurring in HIV disease, I think one would have to be quite cautious in such an extrapolation. Clearly what is needed is a study in HIV patients which measures both extra- and intra-cellular concentrations of all the different vitamin compounds/metabolites, and related to viral load.
- Tryptophan deficiency is stated to be secondary to metabolic shunting towards NAD synthesis. However, it may also be due to increased protein synthesis as a result of the catabolic state, particularly in patients with a high viral load.
- The demonstration of improved clinical outcome with niacin (although only significant in one study) may have been due to confounding factors. In the study by Tang et al, benefit was also found with all the other vitamin B compounds. Should these also be given to patients, or should it only be niacin?

EXHIBIT D

The article by Dr. Murray was read and while it presents some ideas that might have some modifying effects on HIV infection in some niacin-depleted individuals, the importance of this theory does not appear to be substantiated in any clinical trials. Furthermore, many HIV infected people take vitamin supplements and there have never been reports on any observed major effect on the clinical course except in the case of vitamin A. Many individuals and researchers have ideas that vitamins may provide some clinical benefit in HIV infection but the niacin theory needs much better substantiation and of course some kind of clinical trial. Thus, at this point the concept is so purely speculative that this paper should not have a high priority for publication in *The Lancet*.

Review

Safety of high-dose nicotinamide: a review

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Abstract

Nicotinamide, the amide derivative of nicotinic acid, has over the past forty years been given at high doses for a variety of therapeutic applications. It is currently in trial as a potential means of preventing the onset of Type I (insulin-dependent) diabetes mellitus in high-risk, first-degree relatives. Nicotinamide is for regulatory purposes classed as a food additive rather than a drug and has not therefore required the formal safety evaluation normally expected of a new therapy. Because the safety of treatment with megadoses of vitamins cannot be assumed, a full literature review has been undertaken. The therapeutic index of nicotinamide is wide but at very high doses reversible hepatotoxicity has been reported in animals and humans. Minor abnormalities of liver enzymes can infrequently occur at the doses used for diabetes prevention. There is no evidence of teratogenicity from animal studies and nicotinamide is not in itself onco-

genic; at very high doses it does however potentiate islet tumour formation in rats treated with streptozotocin or alloxan. There is no evidence of oncogenicity in man. Growth inhibition can occur in rats but growth in children is unaffected. Studies of its effects on glucose kinetics and insulin sensitivity are inconsistent but minor degrees of insulin resistance have been reported. The drug is well tolerated, especially in recent studies which have used relatively pure preparations of the vitamin. Experience to date therefore suggests that the ratio of risk to benefit of long-term nicotinamide treatment would be highly favourable, should the drug prove efficacious in diabetes prevention. High-dose nicotinamide should still, however, be considered as a drug with toxic potential at adult doses in excess of 3 gm/day and unsupervised use should be discouraged. [Diabetologia (2000) 43: 1337–1345]

Keywords Type I diabetes, nicotinamide, prevention.

Introduction

High-dose nicotinamide therapy has protective effects on beta-cell survival and function in response to a range of toxic and immune stimuli in animal and

in vitro models. Potential therapeutic benefits might be related to its actions as a free radical scavenger, to its availability as a component of the coenzyme nicotinamide adenine dinucleotide (NAD), or to partial inhibition of the nuclear DNA repair enzyme poly(ADP)-ribose polymerase (PARP) which also modulates major histocompatibility complex (MHC) class II expression and apoptosis [1–3]. Nicotinamide also inhibits ADP-ribosyl transferring enzymes modulating immune cell function and survival [3]. It has been tested in a number of human studies as a possible means of preserving beta-cell survival after diagnosis of Type I (insulin-dependent) diabetes mellitus and is currently undergoing large scale evaluation in controlled trials in first-degree relatives at high-risk

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Abbreviations: PARP, Poly(ADP)-ribose polymerase; 2pyr, N-methyl-2-pyridone-5-carboxylic acid amide; ICA, islet cell antibody; ENDIT, European Nicotinamide Diabetes Intervention Trial.

* see Acknowledgements

EXHIBIT E

of progression to Type I diabetes. Although high-dose nicotinamide has a good safety record in human studies, massive doses could in some situations have teratogenic, oncogenic and growth retarding effects in animals. These data are reviewed and the potential risks of high-dose nicotinamide in the attempted prevention of Type I diabetes are outlined.

Niacin, first isolated from rice bran in 1911 and more commonly known as Vitamin B₃, is a water soluble vitamin with a recommended daily allowance of $0.3 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$. It was later recognised to have two distinct but chemically related components, nicotinamide and nicotinic acid. Its deficiency causes pellagra in man. Nicotinamide, first isolated from horse erythrocytes in 1935 [4], is the amide derivative of nicotinic acid. It is a bitter tasting, white, odourless, crystalline powder. The structure consists of a pyridine ring with an amide group in position three. Nicotinamide is a component of NAD, a coenzyme involved in many cellular oxidation-reduction reactions. Despite similarities in chemical structure, nicotinamide and nicotinic acid have very different actions and clinical uses.

Nicotinamide or nicotinic acid or both have been used for many years at high doses in the attempted treatment of a variety of disorders [5–12]. Recent attention has focused on the possibility that nicotinamide might have useful actions in preserving beta-cell function before or after diagnosis of Type I diabetes [13–20]. Its safety in otherwise healthy children and adults is clearly of major importance. The question of dosage needs particular emphasis. The recommended daily intake is about 20 mg (0.2 mmols) a day for an adult or around $0.3 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$. In contrast, the dose used in diabetic and prediabetic patients has ranged from $25\text{--}50 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$ ($1.75\text{--}3.5 \text{ g/day}$). Such pharmacological doses clearly require toxicological scrutiny.

Knowledge of the potential toxicity of nicotinamide is based on a wide range of animal studies and relatively extensive use of high-dose nicotinamide treatment in humans. It is, however, classed as a food additive rather than a pharmaceutical agent and has never undergone the full formal safety evaluation routinely expected of new therapeutic agents. The literature on nicotinamide, although extensive, spans more than 50 years. This has complicated evaluation because, for example, early studies often confuse the side effects of nicotinic acid and nicotinamide, and mixtures have occasionally been used. Further, the purity of the nicotinamide preparations can vary considerably and some preparations include small amounts of nicotinic acid.

Pharmacokinetics of nicotinamide

The pharmacokinetics of nicotinamide are dependent on dose, species, sex and route of treatment [21–25] and metabolic pathways differ according to the species studied [22, 23, 26]. Nicotinamide is readily absorbed parenterally and from all parts of the gastrointestinal tract [4] and peak concentrations are achieved in humans within about 1 hour of oral ingestion of standard preparations [27]. Nicotinamide disappears rapidly from the circulation and is distributed in all tissues. It has a high hepatic extraction ratio and plasma clearance can be reduced in patients with hepatic insufficiency.

Nicotinamide can be oxidised to nicotinamide-*N*-oxide, methylated to *N*-methyl-nicotinamide or hydroxylated to 6-hydroxynicotinamide (Fig. 1). There is no evidence that nicotinamide is metabolised to nicotinic acid in rodents or humans [27, 28]. Hepatic methylation using *L*-methionine as a methyl donor is important in the detoxification of nicotinamide [23]. The product of this reaction, *N*-methyl-nicotinamide, is excreted by the kidneys whereas nicotinamide is reabsorbed by the renal tubules [29]. For this reason only small amounts of the unchanged vitamin appear in the urine even at pharmacological doses of nicotinamide. *N*-methyl nicotinamide is oxidised in the liver, a process that is saturated at high circulating concentrations, and the end products are *N*-methyl-2-pyridone-5-carboxylic acid amide (2 pyr) and *N*-methyl-4-pyridone-3-carboxylic acid amide (4 pyr). The ratio of the two metabolites differs with species and sex. In humans 2 pyr is formed exclusively [28]. Nicotinamide oxidation to nicotinamide-*N*-oxide can also be an important pathway at very high doses in humans and other species [23, 30].

Single dose toxicity

The LD₅₀ s.c. of nicotinamide in rats is 1.68 g/kg [31]. The LD₅₀ in mice is estimated as 4.5 g/kg when given orally and 2.5 g/kg when given intravenously [32]. The therapeutic index of the preparation is correspondingly wide.

Repeated dose toxicity

Potential toxic effects of nicotinamide in animal and human studies are summarised in Table 1.

Liver toxicity

Older clinical studies using nicotinic acid or impure preparations of nicotinamide reported relatively frequent liver enzyme abnormalities [6, 33–35] although

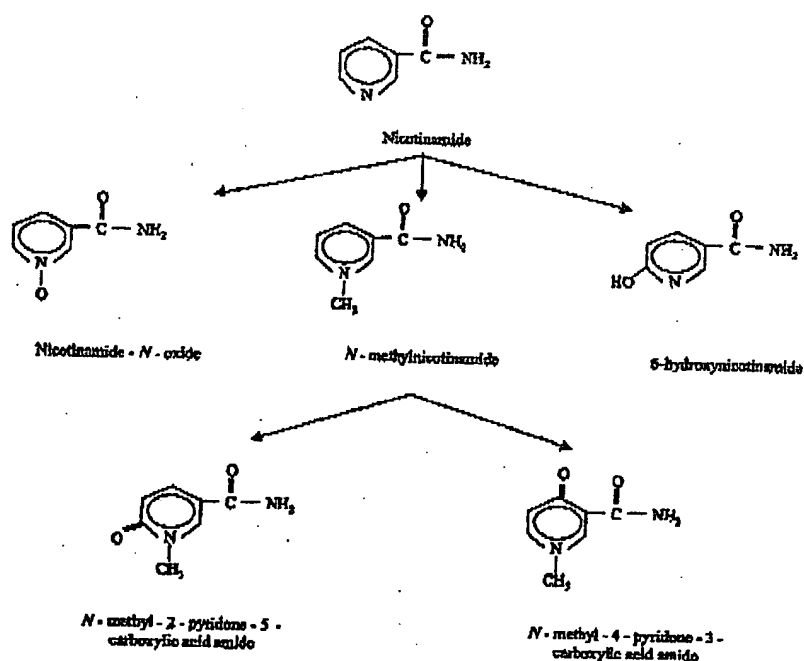


Fig. 1. The pharmacokinetics of nicotinamide [23]

more recent studies using a purified form of nicotinamide have not detected any noteworthy adverse effects on liver enzymes [13, 15, 17, 20, 36]. This experience is described in more detail below.

Nicotinamide at a dose of $500 \text{ mg} \cdot \text{kg}^{-1} \cdot 12 \text{ h}^{-1}$ given i.p. in rats resulted in liver cell enlargement and glycogen deposition with an increase of total hepatic lipids by almost 50%. This effect seemed to be greater in females and was less pronounced when nicotinamide was given orally. Supplementation of the

diet with 0.5% nicotinamide in rats increased liver fatty acids from 4.3 to 15.8%. The addition of 2% nicotinic acid to the diet led to a similar rise from 4.2 to 11.8%. The authors did not, however, examine the livers histologically [25]. These findings have not been confirmed in other studies [37, 38] which found no increase in liver content of fatty acids nor any histological signs of fatty liver degeneration. There is also some evidence that nicotinic acid can protect the liver from the toxic influence of other agents and intraperitoneal injection of the vitamin for 2–3 days at a dose of approximately $300 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$ protected rats against lethal doses of carbon tetrachloride [39].

Table 1. Potential toxic effects of nicotinamide in experimental and human studies

Toxicity	Animal data	Human data
Liver toxicity	0.5% supplementation of diet: increase in liver fatty acids [25], not confirmed by subsequent studies [37, 38]	Jaundice with a frequency of 1/2000 [40]
Teratogenicity	Chick embryos 2.5 mg/egg: coteratogen with acetazolamide [45] 19 mg/egg: inhibition of teratogenic effect of insulin and sulphonamide [42, 44]	No evidence [40]
Oncogenicity	Rodents 350 mg/kg: no detectable carcinogenic action [46] 1% supplementation of drinking water lifelong: no apparent carcinogenic action [47] 305–500 mg/kg: coteratogen for islet cell tumours together with streptozotocin and alloxan [46, 49–51]	No evidence [5–20, 33, 35, 52–57]
Growth-retarding	1% supplementation of diet: growth inhibition in rats [25] but no effect in rabbits or guinea-pigs [26]	No retardation of growth [19, 20]
Insulin response	No evidence	25 mg/kg, 1.2 g/m ² : no effect in normal subjects [61, 62] 25 mg/kg, 1 g/day: improved stimulated C-peptide secretion in newly diagnosed Type 1 diabetic patients [13, 36] 1.2 g/m ² : decreased first-phase insulin response in prediabetic subjects [20]

Assessment of liver toxicity in humans should, as noted, be interpreted with caution because the majority of studies are old and mixtures of nicotinamide and nicotinic acid were often used. In a study on 41 children with attention deficit disorders treated daily for 3 months with a megavitamin regimen comprising 3 g of nicotinamide and ascorbic acid, 1.2 g of calcium pantothenate and 0.6 g of pyridoxine during the last 2 weeks, 17 (42%) subjects showed an increase of serum transaminase concentrations, which in some cases took 4 to 6 weeks to remit [33]. Serum bilirubin values also tended to increase but no child had values above the upper limit of the reference range. It was suggested that nicotinamide was responsible for the liver enzyme abnormalities but as the vitamins were not tested separately this could not be concluded with any certainty. A 35-year-old schizophrenic man developed hepatic toxicity after a daily dose of 9 g nicotinamide [34]. The patient presented with nausea and vomiting and had increased serum transaminase and bilirubin concentrations. A liver biopsy showed increased portal fibrosis and cholestasis. After discontinuation of nicotinamide the symptoms disappeared rapidly and all liver function tests returned to normal values in 3 weeks. A nicotinamide challenge was later done in this patient resulting in reappearance of the symptoms and increased transaminase concentrations in less than 2 weeks. This report illustrates that very large doses of nicotinamide can be hepatotoxic in susceptible people.

Hoffer [40] has stated that the incidence of liver damage as indicated by jaundice caused by nicotinamide or nicotinic acid is very low. In a survey by the Committee on Therapy of the American Schizophrenia Foundation three cases of jaundice were reported out of a total number of 6000 patients treated with megadoses of nicotinamide or nicotinic acid. In one of these patients, treated with nicotinic acid 6 g/day, jaundice resolved when simultaneous phenothiazine treatment was discontinued and in another patient jaundice cleared although treatment with nicotinic acid was resumed. Altschul was reported as finding only four cases of jaundice in a complete literature review [40], and two of these patients were being treated with slow release nicotinic acid. In the Coronary Drug Project minor increases of serum glutamate oxaloacetate transferase and serum alkaline phosphatase were observed in 1119 subjects receiving nicotinic acid 3 g daily for 5 years [35]. Based on his experience over 6 years of treatment with nicotinic acid, Parsons [6] suggested that abnormal liver function tests do not indicate hepatocellular damage but represent changes in liver enzymes, which are rapidly reversible when the drug is discontinued.

One human study has reported that high-dose nicotinamide was able to protect against short-term hepatotoxic effects produced by drinking large amounts of ethanol in white wine [41]. The oxidation

of ethanol leads to an increase in the NADH:NAD ratio which secondarily decreases the activity of key enzymes on ATP-producing pathways and so decreases the production of albumin and fibrinogen by the liver. When nicotinamide is given with ethanol and a standard meal it counteracts this effect and so restores the meal-induced increase in albumin and fibrinogen.

Other toxic effects

No hyperuricaemic effect has been reported during nicotinamide treatment. In contrast nicotinic acid increases uric acid concentrations in some subjects but attacks of gout are extremely rare [40]. Other toxic effects associated with the use of high doses of nicotinic acid are bullous lesions of the skin, toxic amblyopia and hypotensive reactions [32]. No such effects have been reported in connection with megadoses of nicotinamide.

Reproduction

The teratogenic effects of nicotinamide have been studied in chick embryos by injection of the vitamin into the yolk sack of the egg in doses of 2–19 mg/egg. These experiments provide no evidence that nicotinamide is teratogenic by itself [42–44], although it did increase the frequency of acetazolamide-induced malformations in the anterior parts of the embryo from 37.6 to 57.1% at a dose of 2.5 mg/egg [45]. In contrast, nicotinamide at a dose of 19 mg/egg decreased the rate of insulin-induced malformations from 41.7 to 5.9% [42] and was also shown to decrease the rate of sulphonamide-induced malformations [44]. Hoffer reports that Altschul gave nicotinic acid to rabbits before mating and through gestation without harmful effect and he himself gave nicotinic acid to pregnant patients without untoward consequences. He even suggests large doses can prevent some forms of embryonal damage due to a deficiency of NAD [40].

Oncogenic potential

Because nicotinamide potentially promotes survival of cells with DNA damage, possible oncogenicity is an issue of concern, particularly when long-term use of the drug is considered in children and adolescents. Nicotinamide does not seem to have any oncogenic effect when given alone. Two intraperitoneal injections of nicotinamide at a dose of 350 mg · kg⁻¹ · injection⁻¹ when given alone did not result in any detectable carcinogenic action in adult rats [46]. In another study giving 1% nicotinamide in drinking water

to Swiss mice from 6 weeks of age continuously and lifelong had no oncogenic effect [47]. Nicotinamide can however potentiate the ability of streptozotocin or alloxan to induce pancreatic islet cell tumours in rodents [46, 48–51]. The dose of nicotinamide used in these experiments was high, ranging from 305 to 500 mg/kg. There has been no suggestion of oncogenic effects in humans despite high-dose treatment of more than 2000 people in studies dating back over many years [5–20, 33, 35, 52–57]. The quality and completeness of follow-up in these reports is however variable. It is therefore most important that future studies of nicotinamide in prediabetes where DNA damage has already occurred include careful long-term follow-up for evidence of oncogenicity.

Effects on growth

Growth inhibition in rats was first shown in 1942, after inclusion of 1% nicotinamide in the diet. This effect, which was not seen with nicotinic acid, was completely reversed by inclusion of methionine in the diet [25]. No effect on growth was found in young rabbits and guinea-pigs [26]. Inhibition of growth in rats might be due to increased synthesis of *N*-methyl nicotinamide, resulting in a methionine and hence choline deficiency; the effects of paracetamol upon growth in rodents have a similar basis [58]. If so the species difference is easily explained, as methylation to *N*-methyl nicotinamide is not the major route of metabolism in the rabbit or guinea-pig. The unpleasant taste of nicotinamide in solution might explain why food intake was reduced by almost half in nicotinamide-treated animals in one study [25].

A low methionine diet does not affect hepatic methylation in humans [59] and nicotinamide has not been shown to affect growth in children. When height and weight were monitored in 173 children under the age of 12 who were positive for islet cell antibodies (ICA) and were treated with nicotinamide (1 g daily) and for whom two or more readings for height and weight were available, a regression across time showed no change in standard deviation units of height, suggesting that linear growth was not affected [19]. The Deutsche Nicotinamide Intervention Study (DENIS) found no retardation of body growth in 25 ICA-positive children aged 3–12 years and treated with nicotinamide for a median of 2.1 years compared with 30 ICA-positive children of similar age who were treated with placebo [20]. The interim analysis of the safety committee for the ongoing European Nicotinamide Diabetes Intervention Trial (ENDIT), which reviewed data on 734 patient-years of follow-up for 331 ICA-positive family members under the age of 20 taking nicotinamide $1.2 \text{ g} \cdot \text{m}^{-2} \cdot \text{day}^{-1}$ or placebo has reported no concerns regarding growth (ENDIT review committee, personal communication).

Effects on glucose kinetics and insulin secretion

Nicotinamide has no effect in vitro using human islets [60]. Glucose kinetics or basal or stimulated insulin concentrations are unaffected in healthy subjects [61, 62]. In contrast nicotinic acid can induce insulin resistance and glucose intolerance [40]. In patients with recently diagnosed diabetes a meta-analysis of 10 randomised controlled trials found that basal C-peptide concentrations were higher in patients receiving nicotinamide than in those receiving placebo 12 months from diagnosis [63]. Published studies of nicotinamide treatment in people at increased risk of developing diabetes have produced varying results. In one small study insulin sensitivity decreased after two weeks of nicotinamide, although basal and stimulated insulin secretion were unchanged [64], whereas DENIS found a decreased first-phase insulin response in nicotinamide-treated people at 2 years [20].

Tolerability

Nicotinamide is unpalatable when taken in solution and is usually given in capsules or in tablet form. At high doses older studies have reported an overall frequency of side effects of less than 5% [40, 65]. Potential side effects from the older literature are listed in Table 2. In contrast to nicotinic acid, nicotinamide is not a vasodilator and rarely produces cutaneous flushing [66]. Other mucocutaneous adverse effects occurred in less than 1% of subjects. Heartburn is a rare side effect of nicotinamide, and other gastrointestinal symptoms include vomiting, flatulence, soft stools and diarrhoea. Vomiting is rarer with nicotinamide than with nicotinic acid but the incidence of nausea and vomiting can increase during viral infections. Mild headache and dizziness have been reported after giving nicotinamide parenterally [67]. After

Table 2. Side effects of nicotinamide and available data on their frequency when using high doses of the vitamin [40, 65, 69]

Side-effect	Reported frequency
Flushing	≤ 1.5%
Facial erythema	≤ 0.5%
Hives	≤ 0.4%
Sore mouth	≤ 0.4%
Dull headache	≤ 0.5%
Heartburn	≤ 1.6%
Nausea (with radiotherapy)	17–65%
Nausea (without radiotherapy)	≤ 1.5%
Other gastrointestinal symptoms	≤ 0.8%
Inability to focus the eyes	≤ 0.4%
Dry hair	≤ 0.4%
Fatigue	≤ 0.4%

Table 3. Experience with use of megadoses of nicotinamide in different diseases

Year	Indication	Number of people		Dosage	Time Exposed	Comments	Reference
		nicotinamide	placebo				
1987	Newly diagnosed Type I diabetes	7	9	3 g/day	6 months	Remission rate assessed	14
1989	Newly diagnosed Type I diabetes	10	10	1 g/day	45 days		13
1989	Newly diagnosed Type I diabetes	23		200 mg/day	12 months	Open trial	53
1989	Type I diabetes with residual insulin secretion	11	12	3 g/day	9 months	Average 2 years post diagnosis	15
1990	Newly diagnosed Type I diabetes	18	17	100 mg/year old (max 1.5 g/day)	12 months		17
1992	Newly diagnosed Type I diabetes	29	20	40 mg/kg	6 months		16
1995	Newly diagnosed Type I diabetes	28	28	25 mg/kg	12 months		36
1999	Newly diagnosed Type I diabetes	38 36		25 mg/kg 50 mg/kg	12 months	comparison of 25 mg/kg with 50 mg/kg of nicotinamide	70
1991	At high risk of Type I diabetes	14	8	150-300 mg/year old (max 3 g/day)	17 months mean		18
1996	At high risk of Type I diabetes/general population	173	48335o 13463r	1 g/day	30 months	Schoolchildren. Placebo group includes controls (c) + refusals (r)	19
1998	At high risk of Type I diabetes	25	30	1.2 g/m ²	2.1 years median		20
2003	ENDIT	276	276	1.2 g/m ²	Up to 5 years		
1983	Granuloma annulare	1		1.5 g/day	6 months		12
1986	Polymorphous light eruption	42		3 g/day	2 weeks	Open trial	56
1988	Necrobiosis lipoidica	13		1.5 g/day	> 1 month	Open trial	9
1998	Pemphigoid	8		2.5-3 g/day	6 months	Added to minocycline	57
1952-1969	Schizophrenia	982		1.5-6 g/dy	?	Nicotinamide or nicotinic acid given	40
1968	Schizophrenia	262		3 g/day	3-36 months mean 9 months	Personal communication	40
1970	Schizophrenia	17 16 T	24	1 g/23 kg	6 months	16 given tranquilisers (T) with nicotinamide	8

max = maximum

treatment for 2 weeks, side effects of flushing, skin sensations and gastrointestinal symptoms usually resolve [67]. The majority of reported side effects have been reversible after discontinuation of the drug [68]. These findings should be interpreted with some caution because, as indicated earlier, few studies have used pure preparations of nicotinamide.

Experience with use of megadoses of nicotinamide in different diseases

The following studies are summarised in Table 3.

Schizophrenia

Nicotinamide and nicotinic acid were used as adjunctive treatments in a number of psychiatric conditions in studies dating back to the late 1940 s. Hoffer was a strong advocate of its use in the management of schizophrenia and collected data on over 1000 patients who were given nicotinamide or nicotinic acid (1.5-6 g/day) for 3 months to 5 years duration [40]. The American Schizophrenia Foundation also collected information on the use of megavitamin therapy (including nicotinamide) for more than 2 years on 6000 patients and reported an overall incidence of

side effects of less than 5 per cent, with none considered major [40].

Skin conditions

Beneficial effects have been described in over 60 case reports where nicotinamide has also been used in the treatment of polymorphous light eruptions, necrobiosis lipoidica and pemphigoid [9, 12, 56, 57]. Doses ranged from 1.5–3 g/day given for durations of 2 weeks to 6 months with few adverse effects.

Radiotherapy

More recently megadoses of nicotinamide have been used in clinical studies in combination with accelerated radiotherapy and carbogen (ARCON) to radiosensitize inoperable tumours. Its benefit in this situation is apparently due to increased blood flow through the tumour region. Nicotinamide (80 mg/kg, maximum 6 g) is given 1 hour before radiotherapy and continued for the duration of each course of radiotherapy. Preliminary reports from a research workshop using ARCON showed impressive improvements in local control of inoperable head and neck tumours and T3 bladder tumours compared with historical data [69]. Patients undergoing accelerated radiotherapy for head and neck tumours receive nicotinamide at very high doses of 80 mg · kg⁻¹ · day⁻¹ (6 g maximum) and frequently report nausea [69]. This usually occurs within 1 week of starting treatment and is difficult to control with anti-emetics. In patients with unacceptable nausea and vomiting a 25% dosage reduction made symptoms more manageable and symptoms stopped on cessation of therapy.

Type I diabetes

Adjunctive nicotinamide treatment has been reported in 200 people with newly diagnosed Type I diabetes or in longer duration patients with residual insulin secretion. Doses have ranged from 200 mg/day to 50 mg/kg (equivalent 3.5 g/day) for 12 months [13–17, 36, 53, 70]. Only one minor clinical and no biochemical adverse events were noted in these studies.

Nicotinamide has also been used in 200 people at high risk of developing Type I diabetes at doses of 1 g/day to 3 g/day for 4 months to 4 years [18–20]. In addition ENDIT is currently conducting a placebo controlled trial of nicotinamide in family members at high risk of developing the disease. Impurities present in commercial preparations of nicotinamide were removed by crystallisation use in ENDIT. Data are now available for a total of 1626 years of follow-up on 552 people, half of whom have been taking nic-

otinamide at a dose of 1.2 g/m² (maximum 3 g/day). The peak serum concentration obtained with this dose is 100–120 µmol/l which in vivo gives 50% inhibition of PARP [74]. Compliance will be assessed at the end of the trial by measurement of the urinary metabolite of nicotinamide, 2 pyr, in early morning urine samples taken at 6 monthly intervals [71]. Plasma concentrations of 2 pyr can also be measured [28]. Episodes of mildly abnormal liver biochemistry have been reported in 23 subjects receiving either nicotinamide or placebo and in 14 they resolved spontaneously while the participant continued in the trial on unchanged medication. Of the remainder, 7 are still being monitored and 2 have been withdrawn from the trial. There is no apparent difference in the number of abnormal liver function tests between placebo and nicotinamide groups.

Conclusions

Nicotinamide has been used at pharmacological doses in many people over many years with a low incidence of side effects and toxicity. Safety data have not however been collected in a systematic manner and many older reports failed to distinguish between nicotinamide, nicotinic acid and combined vitamin regimens containing nicotinamide. More recent studies have used purer preparations of nicotinamide and toxic effects have been mild and infrequent. In most situations nicotinamide has been used up to a maximum dose of 3.5 g/day but higher doses (6 g/day) used in combination with radiotherapy and carbogen breathing do result in nausea [69]. We have noted a single report of severe but reversible hepatotoxicity in a patient taking 9 g/day of nicotinamide [34]. Hepatic toxicity has occurred in patients taking sustained release formulations of nicotinic acid in dosages of 3 g/day or more [72] although more recently a long-term study of extended release nicotinic acid has found that it is safe when given in dosages of 3 g/day or less [73]. Nicotinic acid combined in a wax matrix vehicle for sustained release has also been shown to be safe with an improved side effect profile [5]. This wax matrix method of producing sustained release tablets is similar to the sustained release nicotinamide preparation used in ENDIT.

Nicotinamide has no teratogenic or oncogenic effects when given alone but has been noted to potentiate the oncogenicity of streptozotocin, although at doses much higher than those used for human studies. It affects growth in rodents but there is no evidence that it has adverse effects on growth in children. The previous literature provides considerable evidence that nicotinamide is a safe therapy to use when given at adult doses of no more than 3 g/day. After careful review of available data the ENDIT study was started in 1994 [74]. Nicotinamide or placebo have been giv-

en in double blind fashion at doses of up to 3 g/day and are well tolerated in the few side effects. The results of ENDIT will be reported in 2003. Should nicotinamide prove efficacious in diabetes prevention, experience to date suggests that the ratio of risk to benefit of long-term treatment would be highly favourable. Long-term surveillance of the study cohort will however be undertaken whatever the outcome of the trial. Until then we continue to advocate caution regarding the unsupervised use of nicotinamide obtained "over the counter". Higher doses of nicotinamide should still be considered as having toxic potential.

Acknowledgements. ENDIT is funded by the European Union concerted action number BMH4-CT96-0771 and by Novo Nordisk. The ENDIT group: *National coordinators:* Austria: E. Schober, Belgium: F. Gorus, Canada: J. Dupre, Croatia: V. Profozic, Denmark: J. Reimers, England: P. Bingley, Finland: M. Knip, France: C. Levy-Marchal, Germany: C. Jaeger, Greece: C. Bartsocas, Hungary: M. Gyorko, Italy: P. Pozzilli, Northern Ireland: D. Carson, Norway: G. Joner, Poland: I. Kinalska, A. Mrozikiewicz, Russia: E. Schwarz, Scotland: S. Green, Spain: A. de Leiva, M. Serrano-Rios, Sweden: J. Ludvigsson, Switzerland: E. Schoenle, Texas: B. Riley, Turkey: Temel Yilmaz. *Review Committee:* A. Drash, O. Aagene, G. Dahlquist, A. Laupacis, A. MacLean, J. Nerup, B. Weber.

References

- Mandrup-Poulsen T, Reimers JJ, Andersen HU et al. (1993) Nicotinamide treatment in the prevention of insulin-dependent diabetes mellitus. *Diabetes Metab Rev* 9: 295-309
- Burkart V, Wang ZQ, Radons J et al. (1999) Mice lacking the poly (ADP-ribose) polymerase gene are resistant to pancreatic beta-cell destruction and diabetes development induced by streptozocin. *Nat Med* 5: 314-319
- Kolb H, Burkart V (1999) Nicotinamide in Type 1 Diabetes. *Diabetes Care* 22: B16-B20
- Marcus R, Coulston AM (1996) Water-soluble vitamins. The vitamin B complex and ascorbic acid. In: Goodman-Gilman A (ed) *The pharmacological basis of therapeutics*. 9th edn. McGraw-Hill, New York, pp 1555-1571
- Koenen JM, Fontaine PL, Wenz JB, Myers S, Huang Z, Ripsin CM (1991) Niacin revisited: A randomized, controlled trial of wax-matrix sustained-release niacin in hypercholesterolaemia. *Arch Intern Med* 151: 1424-1432
- Parsons WB (1961) Studies of nicotinic acid use in hypercholesterolaemia. *Arch Intern Med* 107: 85-99
- Alderman JD, Pasternak RC, Sacks FM, Smith HS, Monrad ES, Grossman W (1989) Effect of a modified, well-tolerated niacin regimen on serum total cholesterol, high density lipoprotein cholesterol and the cholesterol to high density lipoprotein ratio. *Am J Cardiol* 64: 725-729
- Greenbaum GH (1970) An evaluation of nicotinamide in the treatment of childhood schizophrenia. *Am J Psychiatry* 127: 129-132
- Handfield-Jones S, Jones S, Peachey R (1988) High-dose nicotinamide in the treatment of necrobiosis lipoidica. *Br J Dermatol* 118: 693-696
- Hoffer A (1971) Megavitamin B-3 therapy for schizophrenia. *Can Psychiatr Assoc J* 16: 499-504
- Ramsay RA, Ban TA, Lehmann HE, Saxena BM, Bennett J (1970) Nicotinic acid as adjuvant therapy in newly admitted schizophrenic patients. *Can Med Assoc J* 102: 939-942
- Ma A, Medenica M (1983) Response of generalized granuloma annulare to high-dose niacinamide. *Arch Dermatol* 119: 836-839
- Mendola G, Casamitjana R, Gomis R (1989) Effect of nicotinamide therapy upon B-cell function in newly-diagnosed Type 1 (insulin-dependent) diabetic patients. *Diabetologia* 32: 160-162
- Vague P, Viallettes B, Lassman-Vague V, Vallo JJ (1987) Nicotinamide may extend remission phase in insulin-dependent diabetes. *Lancet* i: 619-620
- Vague P, Picq R, Bernal M, Lassman-Vague V, Viallettes B (1989) Effect of nicotinamide on the residual insulin secretion of Type 1 (insulin-dependent) diabetic patients. *Diabetologia* 32: 316-321
- Lewis CM, Canafix DM, Sprafka JM, Barbosa JJ (1992) Double-blind randomized trial of nicotinamide on early-onset diabetes. *Diabetes Care* 15: 121-123
- Chase HP, Butler-Simon N, Garg S, McDuffie M, Hoops SL, O'Brien D (1990) A trial of nicotinamide in newly diagnosed patients with Type 1 (insulin-dependent) diabetes mellitus. *Diabetologia* 33: 444-446
- Elliott RB, Chase HP (1991) Prevention or delay of Type 1 (insulin-dependent) diabetes mellitus in children using nicotinamide. *Diabetologia* 34: 362-365
- Elliott RB, Filcher CC, Fergusson DM, Stewart AW (1996) A population based strategy to prevent insulin-dependent diabetes using nicotinamide. *J Pediatr Endocrinol Metab* 9: 501-509
- Lampeter EF, Klinghammer A, Scherbaum WA et al. (1998) The Deutsche Nicotinamide Intervention Study: an attempt to prevent Type 1 diabetes. *Diabetes* 47: 980-984
- Gluecksohn-Waelsch S, Greengard P, Quinn GP, Teicher LS (1966) Genetic variations of an oxidase in mammals. *J Biol Chem* 242: 1271-1273
- Petlitz WA, Rosen P, Pearson FB (1950) Comparative studies in niacin metabolism. The fate of niacin in man, rat, dog, pig, rabbit, guinea-pig, goat, sheep and calf. *Nutrition* 40: 453-469
- Fumagalli R (1971) Pharmacokinetics of nicotinic acid and some of its derivatives. In: Gey KF, Carlson LA (eds) *Metabolic effects of nicotinic acid and its derivatives*. Hans Huber Publishers, Bern, pp 33-50
- McCannor GM, Bender DA (1986) The metabolism of high intakes of tryptophan, nicotinamide and nicotinic acid in the rat. *Br J Nutr* 56: 577-586
- Handler P, Dann WJ (1942) The inhibition of rat growth by nicotinamide. *J Biol Chem* 146: 357-368
- Handler P (1944) The effect of excessive nicotinamide feeding on rabbits and guinea-pigs. *J Biol Chem* 154: 203-206
- Petley A, Macklin B, Renwick AG, Wilkin TJ (1993) The pharmacokinetics of nicotinamide in humans and rodents. *Diabetes* 44: 152-155
- Gillmor HA, Bolton CH, Hopton M et al. (1999) Measurement of nicotinamide and N-methyl-2-pyridone-5-carboxamide in plasma by high performance liquid chromatography. *Biomed Chromatogr* 13: 360-362
- Beyer KH, Russo HF, Gass SR, Wilhoite KM, Pitt AA (1950) Renal tubular elimination of N-methylnicotinamide. *Am J Physiol* 160: 311-320
- Stratford MR, Dennis MF (1992) High performance liquid chromatographic determination of nicotinamide and its metabolites in human and murine plasma and urine. *J Chromatogr* 582: 145-151
- Anonymous (1989) Niacinamide. In: Budavari S (ed) *The Merck Index*. 11th edn Merck and Co, Rahway, pp 1025-1026
- Hoffer A (1967) Biochemistry of nicotinic acid and nicotinamide. *Psychosomatics* 8: 95-100
- Haslam RH, Dalby JT, Rademaker AW (1984) Effects of megavitamin therapy on children with attention deficit disorders. *Pediatrics* 74: 103-110
- Winter SL, Boyer JL (1973) Hepatic toxicity from large doses of vitamin B3 (nicotinamide). *N Engl J Med* 289: 1180-1182

35. The Coronary Drug Project Research Group (1975) Clofibrate and niacin in coronary heart disease. *JAMA* 231: 360-381
36. Pozzilli P, Visalli N, Signore A et al. (1995) Double blind trial of nicotinamide in recent-onset IDDM (the IMDIAB III study). *Diabetologia* 38: 848-852
37. Janes RG (1953) Certain metabolic effects of niacin and prisco-line. *Am J Physiol* 174: 46-48
38. Nath N, Harper AB, Elvehjem CA (1959) Diet and cholesterol IV. Effects of carbohydrate and nicotinic acid. *Proc Soc Exp Biol Med* 102: 571-574
39. Gallagher CH, Simmonds RA (1959) Prophylaxis of poisoning by carbon tetrachloride. *Nature* 184: 1407-1408
40. Hoffer A (1969) Safety, side effects and relative lack of toxicity of nicotinic acid and nicotinamide. *Schizophrenia* 1: 78-87
41. Volpi E, Lucidi P, Cruciani G et al. (1997) Nicotinamide counteracts alcohol-induced impairment of hepatic protein metabolism in humans. *J Nutr* 127: 2199-2204
42. Landauer W (1948) The effect of nicotinamide and α -ketoglutaric acid on the teratogenic action of insulin. *J Exp Zool* 109: 283-290
43. Uyeki EM, Doull J, Cheng CC, Misawa M (1982) Teratogenic and antiteratogenic effects of nicotinamide derivatives in chick embryos. *J Toxicol Environ Health* 9: 963-973
44. Zwilling E (1949) Reversal of insulin-induced hypoglycaemia in chick embryos by nicotinamide and α -ketoglutaric acid. *Proc Soc Exp Biol Med* 71: 609-612
45. Landauer W, Wakasugi N (1966) Problems of acetazolamide and N-ethylnicotinamide as teratogens. *J Exp Zool* 164: 499-516
46. Rakieten N, Gordon BS, Beatty A, Cooney DA, Davis RD, Schein PS (1971) Pancreatic islet cell tumors produced by the combined action of streptozotocin and nicotinamide. *Proc Soc Exp Biol Med* 137: 280-283
47. Toth B (1983) Lack of carcinogenicity of nicotinamide and isonicotinamide following lifelong administration to mice. *Oncology* 40: 72-75
48. Okamoto H (1985) Molecular basis of experimental diabetes: degeneration, oncogenesis and regeneration of B-cells of islets of Langerhans. *Bioassays* 2: 15-21
49. Yamagami Y, Miwa A, Takasawa S, Yamamoto H, Okamoto H (1985) Induction of rat pancreatic B-cell tumours by the combined administration of streptozotocin or alloxan and poly (ADP-ribose) synthetase inhibitors. *Cancer Res* 45: 1845-1849
50. Kazumi T, Yoshino G, Baba S (1980) Pancreatic islet cell tumours found in rats given alloxan and nicotinamide. *Endocrinol Jpn* 27: 387-393
51. Kazumi T, Yoshino G, Fujii S, Baba S (1978) Tumorigenic action of streptozotocin on the pancreas and kidney in male wistar rats. *Cancer Res* 38: 2144-2147
52. Dumont Herskowitz R, Jackson RA, Soeldner JS, Eisenbarth GS (1989) Pilot trial to prevent Type I diabetes: progression to overt IDDM despite oral nicotinamide. *J Autoimmun* 2: 733-737
53. Pozzilli P, Visalli N, Ghirlanda G, Manna R, Andreani D (1989) Nicotinamide increases C-peptide secretion in patients with recent onset Type I diabetes. *Diabet Med* 6: 568-572
54. Viallettes B, Picq R, du Rostu M, Charbonnel B, Rodier M, Mirotz J, et al. (1990) A preliminary multicentre study of the treatment of recently diagnosed Type I diabetes by combination nicotinamide-cyclosporin therapy. *Diabet Med* 7: 731-735
55. Zackheim HS (1978) Topical 6-aminonicotinamide plus oral niacinamide therapy for psoriasis. *Arch Dermatol* 114: 1632-1638
56. Neumann R, Rappold E, Pohl-Marki H (1986) Treatment of polymorphous light eruption with nicotinamide: a pilot study. *Brit J Dermatol* 115: 77-80
57. Reiche L, Wojnarowska F, Mallon E (1998) Combination therapy with nicotinamide and tetracyclines for cicatricial pemphigoid: further support for its efficacy. *Clin Exp Dermatol* 23: 254-257
58. McLean AE, Armstrong GR, Beales D (1989) Effect of D- or L-Methionine and cysteine on the growth inhibitory effects of feeding 1% paracetamol to rats. *Biochem Pharmacol* 38: 347-352
59. Jenks BH, McKee R, Swendsel MB, Faraji B, Figueroa WG, Clemens RA (1987) Methylated niacin derivatives in plasma and urine after an oral dose of nicotinamide given to subjects fed a low-methionine diet. *Am J Clin Nutr* 46: 496-502
60. Sandler S, Hellerstrom C, Eizirik DL (1993) Effects of nicotinamide supplementation on human pancreatic islet function in tissue culture. *J Clin Endocrinol Metab* 77: 1574-1576
61. Bingley PJ, Cuddeback G, Bonfanti R, Gale EAM (1993) Nicotinamide and insulin secretion in normal subjects. *Diabetologia* 36: 675-677
62. Paul TL, Hramiak JL, Mahon JL et al. (1993) Nicotinamide and insulin sensitivity. *Diabetologia* 36: 369
63. Pozzilli P, Kolb H, Browne PD and the Nicotinamide Trialists (1996) Meta-analysis of nicotinamide treatment in patients with recent-onset IDDM. *Diabetes Care* 19: 1357-1363
64. Greenbaum CJ, Kahn SE, Palmer JP (1996) Nicotinamide's effects on glucose metabolism in subjects at risk for IDDM. *Diabetes* 45: 1631-1634
65. Zackheim HS, Vasily DB, Westphal ML, Hastings CW (1981) Reactions to niacinamide. *J Am Acad Dermatol* 4: 736-737
66. Ranchoff RE, Tomecki KJ (1986) Niacin or niacinamide? Nicotinic acid or nicotinamide? What is the difference? *J Am Acad Dermatol* 15: 116-117
67. Anonymous (1985) Vitamin B complex, niacin. In: McEvoy GK (ed) American hospital formulary service drug information. American Society of Hospital Pharmacists, Bethesda, pp 1685-1687
68. Anonymous (1993) Nicotinic acid, nicotinamide. In: Reynolds JE (ed) The extra pharmacopoeia. 30th edn. Martindale, London, pp 1045-1046
69. Denckamp J, Fowler JP (1997) ARCON - Current status: Summary of a workshop on preclinical and clinical studies. *Acta Oncol* 36: 517-525
70. Visalli N, Cavallo MG, Signore A et al. (1999) A multi-centre randomized trial of two different doses of nicotinamide in patients with recent-onset Type I diabetes (The IMDIAB VI). *Diabetes Metab Res Rev* 15: 181-185
71. Moore WP, Bolton CH, Downs L, Gillmor HA, Gale EAM (2000) Measurement of N-methyl-2-pyridone-5-carboxamide in urine by high performance liquid chromatography. *Biomed Chromatogr* 14: 69-71
72. Rader AI, Calvert RJ, Hathcock JN (1992) Hepatic toxicity of unmodified and time-release preparations of niacin. *Am J Med* 92: 77-81
73. Capuzzi DM, Guyton JR, Morgan JM et al. (1998) Efficacy and safety of an extended-release niacin (niapan): a long-term study. *Am J Cardiol* 82: 74U-81U
74. Potot F, Reimers JJ, Andersen HU (1993) Nicotinamide - biological actions and therapeutic potential in diabetes prevention. IDIG Workshop, Copenhagen, Denmark, 4-5 December 1992. *Diabetologia* 36: 574-576



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SERIAL NUMBER	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.
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07/906,689 06/30/92 MURRAY

M 3176-1

EXAMINER

KUNZ, G

ART UNIT

PAPER NUMBER

1803

2

DATE MAILED: 08/25/92

LEWIS F. GOULD, JR.
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27

This is a communication from the examiner in charge of your application.
COMMISSIONER OF PATENTS AND TRADEMARKS

10-25-92
11-25-92
1-25-93
2-25-93

☒ This application has been examined ☒ Responsive to communication filed on 6/30/92 ☐ This action is made final.

A shortened statutory period for response to this action is set to expire 3 month(s), _____ days from the date of this letter.
Failure to respond within the period for response will cause the application to become abandoned. 35 U.S.C. 133

Part I THE FOLLOWING ATTACHMENT(S) ARE PART OF THIS ACTION:

- | | |
|---|--|
| 1. <input checked="" type="checkbox"/> Notice of References Cited by Examiner, PTO-892. | 2. <input checked="" type="checkbox"/> Notice re Patent Drawing, PTO-948. |
| 3. <input type="checkbox"/> Notice of Art Cited by Applicant, PTO-1449. | 4. <input type="checkbox"/> Notice of Informal Patent Application, Form PTO-152. |
| 5. <input type="checkbox"/> Information on How to Effect Drawing Changes, PTO-1474. | 6. <input type="checkbox"/> _____ |

Part II SUMMARY OF ACTION

1. ☒ Claims 1-8 are pending in the application.

Of the above, claims _____ are withdrawn from consideration:

2. ☐ Claims _____ have been cancelled.

3. ☐ Claims _____ are allowed.

4. ☒ Claims 1-8 are rejected.

5. ☐ Claims _____ are objected to.

6. ☐ Claims _____ are subject to restriction or election requirement.

7. ☒ This application has been filed with informal drawings under 37 C.F.R. 1.85 which are acceptable for examination purposes.

8. ☐ Formal drawings are required in response to this Office action.

9. ☐ The corrected or substitute drawings have been received on _____. Under 37 C.F.R. 1.84 these drawings are ☐ acceptable ☐ not acceptable (see explanation or Notice re Patent Drawing, PTO-948).

10. ☐ The proposed additional or substitute sheet(s) of drawings, filed on _____ has (have) been ☐ approved by the examiner. ☐ disapproved by the examiner (see explanation).

11. ☐ The proposed drawing correction, filed on _____, has been ☐ approved. ☐ disapproved (see explanation).

☐ Acknowledgment is made of the claim for priority under U.S.C. 119. The certified copy has ☐ been received ☐ not been received

☐ been filed in parent application, serial no. _____; filed on _____

EXHIBIT F

This application has been filed with informal drawings which are acceptable for examination purposes only. Formal drawings will be required when the application is allowed. The required changes to the drawings are detailed on the accompanying PTO Form 948.

Claims 1 - 8 are rejected under 35 U.S.C. 101 because the claimed invention lacks patentable utility.

The specifications provide data only for inhibiting HIV in cells in culture. There is no data to substantiate the alleged utility for treating human subjects infected with HIV. There is no detailed regimen or protocol defined that includes dosage, required blood levels, period of treatment, etc.

Without statistically significant data documenting the claimed method for treating patients, the person of ordinary skill in the art, knowing the unpredictability of extrapolating from in vitro results to in vivo performance, would have good reason to doubt efficacy of applicant's invention. The burden falls on the applicant to substantiate his alleged in vivo method of treating humans with nicotinamide.

The following is a quotation of the first paragraph of 35 U.S.C. 112:

The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same and shall set forth the best mode contemplated by the inventor of carrying out his invention.

The specification is objected to under 35 U.S.C. 112, first paragraph, as failing to provide an adequate written description and failing to teach adequately how to make and/or use the invention, i.e. failing to provide an enabling disclosure.

The specifications provide data only for inhibiting HIV in cells in culture. There is no data to substantiate the alleged utility for treating human subjects infected with HIV.

~~There is no detailed regimen or protocol defined that includes dosage, required blood levels, period of treatment, etc.~~

Without statistically significant data documenting the claimed method for treating patients, the person of ordinary skill in the art, knowing the unpredictability of extrapolating from in vitro results to in vivo performance, would have good reason to doubt efficacy of applicant's invention. The burden falls on the applicant to substantiate his alleged in vivo method of treating humans with nicotinamide.

~~Claims 1 and 7 read on a method of treating humans infected with HIV with "a post transcriptional inhibitor of HIV".~~ These claims encompass alleged inhibitors of HIV that the specifications do not even identify by chemical name. Since the method of treatment using nicotinamide is not enabled, certainly these other unnamed agents without even so much as in vitro data are also not enabled.

Claims 1 - 8 are rejected under 35 U.S.C. 112, first

paragraph, for the reasons set forth in the objection to the specification.

Claim 4 is rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

The meaning of " 3 D " should be replaced by the three specific symptoms symbolized by this term.

The Kull, Jr. et al. and White et al. references are cited in order to establish the contemporary knowledge in the art of nicotinamide as a important dietary consitutent and as a wound healing constituent.

No claim is allowed.

Papers related to this application may be submitted to Group 180 by facsimile transmission. Papers should be faxed to Group 180 via the PTO Fax Center located in Crystal Mall 1. The faxing of such papers must conform with the notice published in the Official Gazette, 1096 OG 30 (November 15, 1989). The CM1 Fax Center number is (703) 308-4227.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Examiner Kunz whose telephone number is (703) 308-3995.

A.K.
25 Gary L. Kunz:glk
August 24, 1992

JOHNNIE R. BROWN
SUPERVISORY PATENT EXAMINER
ART UNIT 183

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Michael F. Murray, M.D.

Serial No.: not yet known

Filing Date: submitted herewith

For: HUMAN IMMUNODEFICIENCY VIRUS (HIV)

CERTIFICATE OF EXPRESS MAIL

Hon. Commissioner of Patents and Trademarks
Washington, D.C. 20231

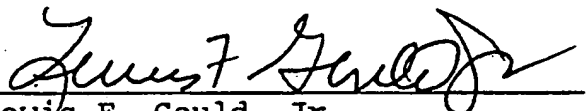
Sir:

I hereby certify that this document, namely the above-identified complete patent application is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicated below and is addressed to the Commissioner of Patents and Trademarks, Washington, D.C. 20231.

"Express Mail" mailing label number TB135927132.

Date of Deposit June 30, 1992.

Respectfully submitted,


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Docket # 3176-1

PATENT APPLICATION TRANSMITTAL LETTER

TO THE COMMISSIONER OF PATENTS AND TRADEMARKS

Docket No. 3176-1

Transmitted herewith for filing of the patent application of _____

Michael F. Murray, M.D.

for METHOD OF INHIBITING HUMAN IMMUNODEFICIENCY VIRUS (HIV)

Enclosed are:

- 5 sheets of drawing (3 sets of photocopies)
- an assignment of the invention to _____
- a certified copy of a _____ application
- associate power of attorney
- X verified statement to establish small entity status under 37 C.F.R. 1.9 and 1.27 (Independent Inventor)
- X Certificate of Express Mail

CLAIMS AS FILED

FOR	NO. FILED	NO. EXTRA
Basic fee		
Total Claims	8 -20-	0
Indep Claims	3 -3-	0
multiple dependent claim present		

If the difference in Col. 1 is less than zero, enter "0" in Col. 2

SMALL ENTITY

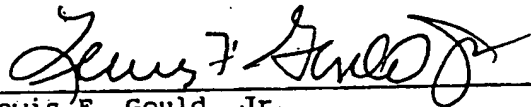
RATE	FEE
	\$ 345
X \$10 =	\$
X \$36 =	\$
X \$110 =	\$
TOTAL	\$ 345

Other than a Small Entity

RATE	FEE
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X \$20 =	\$
X \$72 =	\$
X \$220 =	\$
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- X Any patent application processing fees under 37 C.F.R. 1.17.

Date June 30, 1992


 Lewis F. Gould, Jr.
 Registration No. 25,057

DECLARATION FOR PATENT APPLICATION

Docket: 3176-1

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled METHOD OF INHIBITING HUMAN IMMUNODEFICIENCY VIRUS (HIV), the specification of which

(check one) X is attached hereto.
_____ was filed on _____ as Application Serial
No. _____ and was amended on
_____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose to the Office all known information which is material to patentability as defined in Title 37, Code of Federal Regulation Section 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code 119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

Prior Foreign Application(s)

Priority Claimed

Yes No

(Number) (Country) (Day/Month/Year Filed)

Yes No

30 Number) (Country) (Day/Month/Year Filed)

Yes No

(Number) (Country) (Day/Month/Year Filed)

I hereby claim the benefit under Title 35, United States Code, Section 120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, Section 112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, Section 1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

(Appln. Serial No.) (Filing Date) (Status-patent, pending, abandoned)

(Appln. Serial No.) (Filing Date) (Status-patent, pending, abandoned)

I ~~E~~ereby appoint the following attorney(s) and/or agent(s) to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith:

Walter J. Blenko, Jr., Registration No. 18,526; Karl L. Spivak, Registration No. 18,934; Arnold B. Silverman, Registration No. 22,614; Richard V. Westerhoff, Registration No. 24,454; Lewis F. Gould, Jr., Registration No. 25,057; Stephan P. Gribok, Registration No. 29,643; Robert E. Greenstien, Registration No. 27,556; Suzanne Kikel, Registration No. 28,230; Michael J. Kline, Registration No. 31,632; Craig G. Cochenour, Registration No. 33,666; John V. Silverio, Registration No. 34,014; Robert J. Kapalka, Registration No. 34,198 and George Stacey, Registration No. 35,688.

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I ~~h~~ereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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Full name of second joint inventor, if any _____
Second Inventor's signature _____ Date _____
Residence _____
Citizenship _____
Post Office Address _____

METHOD OF INHIBITING HUMAN IMMUNODEFICIENCY VIRUS (HIV)

Background of the Invention

1. Field of the Invention:

This invention relates to the inhibition of human immunodeficiency virus (HIV) replication, the etiological agent clinically associated with acquired immunodeficiency syndrome (AIDS). More particularly, the invention relates to administering a therapeutically effective amount of niacin, such as nicotinamide, to host cells that are infected with HIV as well as those not infected.

2. Prior Art:

AIDS is the clinical syndrome associated with HIV infection. AIDS already has claimed nearly 300,000 lives. HIV is believed to have infected approximately 10 million people around the world and 40 million people are expected to be infected by the end of the century. In the little more than ten years since the first reported cases of AIDS, a great deal has been learned about this retroviral disease and its diverse manifestations. There remain, however, a number of clinical expressions of AIDS which go unexplained despite the efforts of the medical community to elucidate their etiology.

Viruses, such as HIV, are packets of infectious nucleic acids, the genetic material, surrounded by a protective protein coat. Viruses are unable to generate metabolic energy or to synthesize proteins and thus are characterized by total dependence on living cells for replication and proliferation. It has been discovered that HIV contains the enzyme RNA-

directed DNA polymerase or reverse transcriptase which is required for the synthesis of viral DNA in a living cell and is crucial for HIV replication.

Medical researchers in the past have focused their efforts on the development of anti retroviral agents which inhibit or block reverse transcriptase (RT) activity. Such agents include among others, AZT (3'-azide-3'-deoxythymidine), DDI (2'-3'-dideoxyinosine), and DDC (2'-3'-dideoxycytidine), each of which are thought to block HIV proliferation in cells. However, these RT inhibitors do not cure HIV, do not block HIV replication completely, and they frequently produce undesired side effects.

Other studies have focused on inhibiting protein synthesis in HIV infected cells in a manner which result in killing of those cells. For example, U.S. Patent No. 4,867,976 discloses a method of treatment of HIV using a liposome containing a diphtheria toxin dissociation fragment to specifically inhibit protein synthesis in an HIV infected cell. The diphtheria toxin induces covalent modification and inactivation of the elongation factor necessary for elongation of a peptide chain. The catalyzed reaction involves the cell's nicotinamide adenine dinucleotide (NAD⁺) donating its adenosine diphosphate (ADP) ribose moiety (ADP-Ribose) to the elongation factor with the release of nicotinamide.

Another study of suppression of HIV replication at the transcriptional activation level is Pauling et al., Proc. Natl. Acad. Sci 87:7245, 1990, which discloses that ascorbic acid (vitamin C) is effective to suppress HIV replication in vitro
5 by diminishing viral protein production in infected cells and RT stability in extracellular virons.

Niacin, a component of Vitamin B complex, is a generic term that can apply to both nicotinic acid, i.e., $C_6H_5NO_2$, (pyridine-3-carboxylic acid) or nicotinamide, i.e., $C_6H_5ON_2$ (3-pyridinecarboxamide).
10 Niacin is a precursor to the biosynthesis of nicotinamide adenine dinucleotide (NAD). Nicotinamide adenine dinucleotide participates in a wide array of oxidation-reduction reactions catalyzed by dehydrogenase or oxido-reductase enzymes. Virtually every aspect of cellular
15 metabolism involves NAD/NADH or NADP/NADPH dependent reactions. In absence of sufficient supplies of NAD or niacin precursors for NAD biosynthesis, cellular functions and life itself would be impaired. (DiPalma et al., Annu. Rev. Nutr. 11:169, 1991). It has not been suggested heretofore to use niacin as an HIV
20 inhibitor.

Nicotinamide, the amide of nicotinic acid, is produced in cells as an end product of a number of mono and poly ADP reactions. The reactions are characterized' by:

NAD ADP-Ribose-Protein + Nicotinamide.

This ADP-Ribosylation reaction has the potential to deplete cellular NAD. Nicotinamide is a known end-product and inhibitor of ADP ribosylation reactions.

Since only the intact virus can infect a cell, it is
5 desired to inhibit intact and infective HIV replication. It has not been suggested heretofore that suppression of intact and infective HIV gene expression in host cells of patients infected with HIV can be accomplished with nicotinamide. The present invention provides a method for inhibition of HIV
10 replication in cells infected with the intact virus through administration of a therapeutically effective amount of nicotinamide. The present invention is based on the inventor's clinical observations, knowledge of compound toxicity in humans, and observation of intact virus inhibition.

15 Pellagra is a disease which was first described in 1735. Through the work of Elvehjem et al., J. Am. Chem. Soc. 68:1767, 1937, it became clear that endemic pellagra was caused by nicotinic acid (niacin) deficiency, a precursor to NAD biosynthesis. Classically, the disease was clinically
20 identified by dermatitis, diarrhea, and dementia (3 D's). Nicotinamide replacement has been used to treat pellagra. Furthermore, Nicotinamide, a B complex vitamin, is relatively nontoxic when administered to humans even in substantial quantities, i.e., of up to as much as 5 grams per day.

Clinical observation shows that both pellagrins and AIDS patients demonstrated like symptoms (i.e., the 3 D's). This observation led to the idea that it could be possible to inhibit HIV by administering a therapeutically effective amount of nicotinamide to patients infected with HIV. Surprisingly, it was found that HIV replication is inhibited by administration of nicotinamide in a therapeutically effective dose. The mechanism through which this inhibition occurs is not necessarily understood, although it is theorized that nicotinamide, administered in therapeutically sufficient quantity, may function as an ADP ribosylation inhibitor which serves to suppress HIV gene expression at the post transcriptional level.

SUMMARY OF THE INVENTION

It is the object of the invention to inhibit HIV replication and proliferation through administration of a post transcriptional inhibition of HIV.

5 It is another object of the invention to inhibit adverse effects associated with HIV production.

It is still another object of the invention to inhibit HIV with relatively non-toxic inhibiting agents.

It is another object of the invention to inhibit HIV
10 production by combining the method of this invention with known HIV inhibitors, such as AZT, DDI, DDC, etc.

These and other objects of the invention are achieved by providing a method of administering a therapeutically effective amount of nicotinamide to a patient infected with HIV, the
15 etiological agent clinically associated with AIDS.

The invention is grounded on the observed similarities of clinical expressions of AIDS and pellagrins patients. Prominent among unexplained infirmities exhibited by AIDS patients are seborrheic dermatitis, AIDS enteropathy and AIDS
20 dementia. The inventor's recognition of the striking common symptoms presented by the three D's in pellagra and AIDS patients, led to a possible alternative explanation for some idiopathic AIDS symptoms. The inventor believed and concluded that HIV induces a pellagra-like state through metabolic
25 depletion of niacin, and that the pellagrin like symptoms did

not arise through dietary deficiency. The inventor further believes that HIV replication requires the NAD hydrolysis reaction, i.e., ADP ribosylation, during the post transcription portion of its life cycle which is necessary for HIV
5 replication and proliferation.

The invention, it is believed, makes use of nicotinamide, an inhibitor of ADP ribosylation, to reverse the equilibrium of the NAD depletion reaction and thus suppress the metabolic depletion of niacin, the NAD precursor, thereby inhibiting the
10 HIV replication. Furthermore, others have found that AZT, DDI and DDC prevent HIV replication at a pre transcriptional state (i.e., reverse transcription) and, thus, the combined use of nicotinamide, a post transcriptional inhibitor, with other HIV inhibitors is believed suitable for the effective therapeutic
15 treatment of AIDS.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a graph which shows that cell free HIV production from HIV infected cells is inhibited in dose dependent fashion by increasing millimolar concentrations of nicotinamide measured by the Reverse Transcriptase Assay.

Fig. 2 is a graph which shows that the surviving cells of Fig. 1 exhibited a CD4 marker with increased concentrations of nicotinamide measured by flow cytometry to study the surface markers of the surviving lymphocytes.

Fig. 3 is a graph which shows that inhibition of cell free HIV production from HIV infected cells is specific for nicotinamide and no inhibition of HIV is demonstrated by nicotinic acid, the other form of niacin, or thiamine, another B-complex vitamin measured by the Reverse Transcriptase Assay.

Fig. 4 is a graphs which shows that nicotinamide inhibition of HIV is occurring at the post transcriptional level demonstrated by chronically HIV infected U1 cells with interleukin induced gene expression.

Fig. 5 is a graph which shows that nicotinamide does not inhibit the activity of HIV 1 Reverse Transcriptase.

DETAILED DESCRIPTION OF THE INVENTION

The invention provides a method for treatment of HIV and its clinical effects of the three D's by administering nicotinamide to an HIV infected patient in an amount effective
5 to elicit a therapeutic response through post-transcriptional inhibition of HIV. It is preferred to administer nicotinamide in "pharmacologic doses", i.e., greater than 100 times the recommended daily allowance (RDA), or in an amount of 1 to 5 grams per day. The nicotinamide preferably may be administered
10 in equally divided doses taken approximately every eight hours.

The immediate or short term goal of the invention is to administer sufficient nicotinamide so as to obviate the 3 D symptoms exhibited by a patient. Of course, nicotinamide therapy can be continued indefinitely thereafter to maintain
15 this state. Routine evaluation of red blood cell NAD levels could be made to determine whether sufficient nicotinamide is administered to satisfy established norms.

Another clinical evaluation to be made routinely to confirm administration of sufficient nicotinamide to inhibit
20 HIV is the measurement of viable CD4 lymphocytes. A patient CD4 level of 500 or greater demonstrates HIV inhibition, and is a goal of this invention.

Nicotinamide therapy at dosage levels sufficient to satisfy this invention is safe as well as effective, and is

desired because of its low side effect profile, and its high therapeutic index.

The nicotinamide may be administered orally, parenterally and rectally, and with any pharmaceutically accepted adjuvant or carrier. Laboratory and clinical studies to date have demonstrated utility only for the compound known as nicotinamide. It is postulated however, that nicotinic acid could be found in future clinical studies to produce the desired therapeutic effect.

This invention will be hereinafter explained more in detail by way of examples. However, these examples should not be construed to limit the scope of the invention and are to be understood merely for the purpose of illustration.

EXAMPLE 1 - HIV Infection In Human Lymphocytes

Heparinized blood from a HIV seronegative donor was centrifuged using the Ficoll Hypaque gradient technique to isolate the peripheral blood lymphocytes. The lymphocytes were then suspended in culture media (RPMI-1640 WITH 10% FBS) at a concentration of 2 million cells/ml. The cells were incubated with 5µg/ml of PHA for 72 hours. Following PHA stimulation the cells were divided into 4 culture flasks containing 10 million cells and 100 ng (p24 equivalent) of HIV-Z6. Three hours post infection the cells were washed with phosphate buffered saline, and then resuspended in 10ml of culture media (final concentration of 1 million cells/ml) with 2.5µ/ml of IL2. At

the time of resuspension three of the flasks received varied amounts of nicotinamide (NAM) and one flask was maintained as positive control (without NAM).

Post infection aliquots were taken every 2 days for measurement to virus production. The samples were stored at -20C until the completion of the experiment and then the RT assay was performed on all samples to quantitate the virus production at 2, 4, 6, 8, and 10 days post infection.

In Fig. 1 the abscissa indicates the time post infection in days, and the ordinate indicates the amount of cell free virus produced in each culture. The results demonstrate a dose dependent inhibition of HIV production with 5mM, 10mM and 15mM concentration of NAM.

EXAMPLE 2 - Lymphocytes Subsets In Active HIV Infection

The lymphocytes from Example 1 were taken from culture on post infection day 11 and subject to flow cytometry (Becton Dickinson Lysys II Version 1.0) in an effort to demonstrate the percentage of viable cells in each culture which had detectable CD4 and CD8 surface markers.

A productive HIV infection will decrease the percentage of lymphocytes bearing CD4 marker by two means: cytotoxicity to infected cells and downregulation of the marker in infected cells. In culture the percentage will steadily decrease over time from an original percentage of approximately 60-70%. The loss of CD4+ lymphocytes in patients is the hallmark of

advancing immunosuppression and clinical decline in HIV infection.

In Fig. 2 the abscissa marks the concentrations of NAM added to each culture and the ordinate indicates the percentage of living cells which fluoresced positive for CD4, CD8 or neither (0). Note that a small percentage of cells can fluoresce positive for both CD4 and CD8 and thereby make the total in the column exceed 100%. The results demonstrate that the addition of nicotinamide not only inhibited cell free virus production but also preserved CD4+ lymphocytes in a dose dependant manner.

EXAMPLE 3 - HIV Infection In Human Lymphocytes

The infection of peripheral blood lymphocytes from seronegative donors was carried of in this experiment in a manner identical to that described in Example 1. In this example, however, at the time of resuspension in culture media containing IL2, the four culture flasks were treated with: 5mM nicotinamide, 5mM nicotinic acid, 5mM thiamine, or no addition (positive control).

In Fig. 3 the abscissa indicates the time post infection in days and the ordinate indicates the amount of cell free virus produced in each culture. The results indicate that the inhibition of HIV by nicotinamide cannot be generalized to nicotinic acid (another form of niacin) nor to thiamine (another B-complex vitamin) under these conditions.

EXAMPLE 4 - Post Transcriptional Inhibition of HIV

The U1 cell line, which is chronically infected with two proviral copies of HIV, was used in an experiment taking 4 culture flasks containing U1 cells at a concentration of $1 \times 10^6/\text{ml}$ in 5ml of culture media (RPMI 1640 containing 10% FBS). Three flasks were then either treated with the post transcriptional stimulator IL6 [$10 \mu\text{ml}$] and/or NAM [5mM]; with the fourth flask acting as a negative control. Aliquots were taken at days 4 and 8 post stimulation and were stored at -20C until they were subject to RT Assay.

In Fig. 4 the abscissa indicates the number of days post stimulation, and the ordinate indicates the amount of HIV produced as measured by RT assay. The results indicate that while NAM alone has an inhibitory effect on the low level constitutive production of HIV in the negative control; it has a profound inhibitory effect (IL6/5mM NAM) on the post transcriptional stimulation of HIV production in the positive control (IL6 alone).

EXAMPLE 5 - Reverse Transcriptase Activity

The effect to NAM on the activity of the HIV reverse transcriptase enzyme was assessed in a cell free system to see if this inhibitor also has direct inhibitory effects on this viral enzymes activity.

The reverse transcriptase assay was run in three wells. Each well contained 50 μl of stock solution made of: 10 ml of 1M

Tris(pH7.8), 5 ml of 3M KCl, 4 ml of 0.1M DTT, 6.6ml of 0.15M MgCl, 10ml of 100µg/ml PolyA, 10 ml of 31.5 µ/ml oligo dT, 200µl of 10µCi/ml dttP, 5ml of 2% NP-40 and 149.2 ml of ddH₂O. Two of the wells then had 50 ng (p24 equivalent) of HIV-HXB2 added to them and the experimental well had NAM added to a final concentration of [5mM].

In Fig. 5 the positive control (HIV and Solution) shows no statistical difference from the well with 5mM NAM added, though both are significantly different from the background activity shown in the negative control. This demonstrates the lack of significant inhibition of HIV reverse transcriptase by NAM which further supports the discovery that the inhibitory effect of NAM on HIV is post transcriptional and therefore at a site separate from that where it is taught that compounds currently used to inhibit HIV in infected patients (i.e., - AZT, DDI, and DDC) exert their therapeutic effect.

Studies completed to date have not been sufficient to determine whether the combined nicotinamide (post transcriptional) and AZT, DDI and DDC (RT) therapy results in a cumulative or greater then cumulative effect. It is possible, however, that reduced amounts of either the post transcriptional or RT effective compounds could be administered and achieve the desired therapeutic response or that a greater than expected therapeutic response could be obtained with the

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administration of usual amounts of the post transcriptional and
RT effective compounds.

What is claimed is:

1. A method of inhibiting human immunodeficiency virus (HIV) which comprises:

administering to an HIV infected patient a therapeutically effective amount of a post transcriptional inhibitor of HIV.

2. A method of inhibiting human immunodeficiency virus (HIV) which comprises:

administering nicotinamide to an HIV infected patient in an amount effective to elicit a therapeutic response through post transcriptional inhibition of HIV.

3. The method of claim 2 wherein said nicotinamide is administered in an amount of from about 1 gram to about 5 grams per day.

4. The method of claim 3 wherein the nicotinamide is administered until 3 D symptoms exhibited by a patient are eliminated.

5. The method of claim 3 wherein the nicotinamide is administered in an amount and for a time sufficient to achieve a patient CD4 level of 500 or greater.

6. The method of inhibiting HIV of claim 3, wherein nicotinamide is administered orally, parenterally and rectally.

7. The method of inhibiting HIV which comprises:

administering to an HIV infected patient a therapeutically effective amount of a post transcriptional inhibitor of HIV in combination with a RT inhibitor of HIV.

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8. The method of claim 7 wherein the post transcriptional inhibitor is nicotinamide and the RT inhibitor is a member selected from the group comprising AZT, DDI, DDC and other RT HIV inhibitors.

ABSTRACT OF THE DISCLOSURE

This invention relates to a method of inhibiting human immunodeficiency virus (HIV) which comprises administering a therapeutically effective amount of nicotinamide to HIV
5 infected patients. This invention further relates to post transcriptional inhibition of HIV replication in infected and uninfected cells of a patient with HIV.

Figure 1

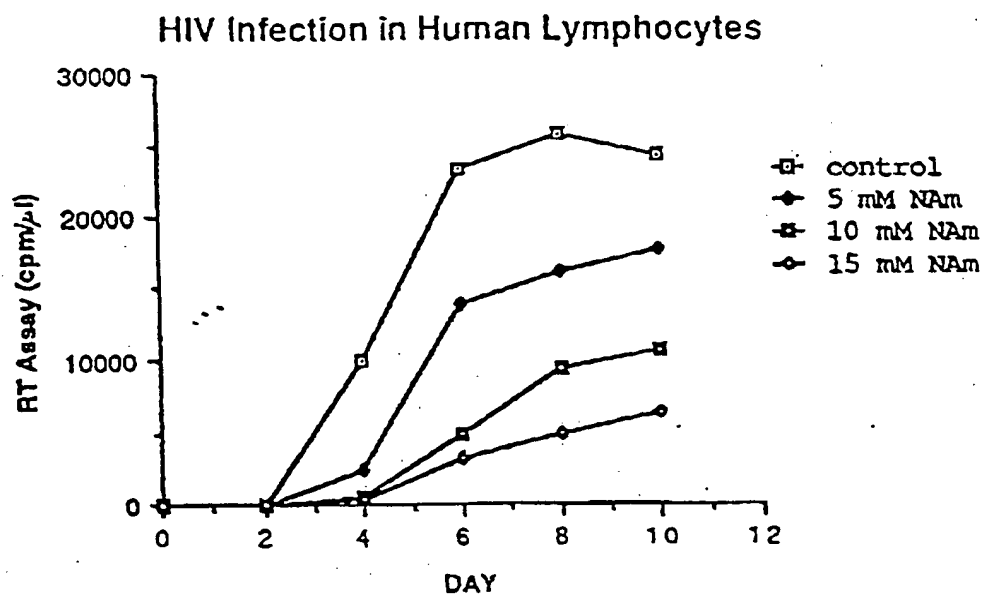


Figure 2

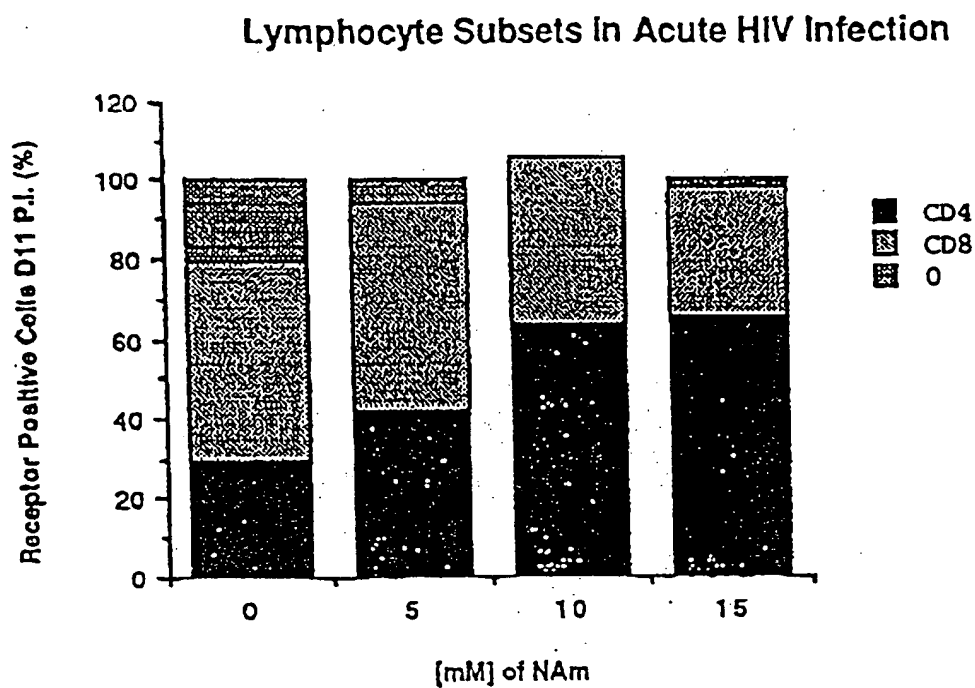


Figure 3

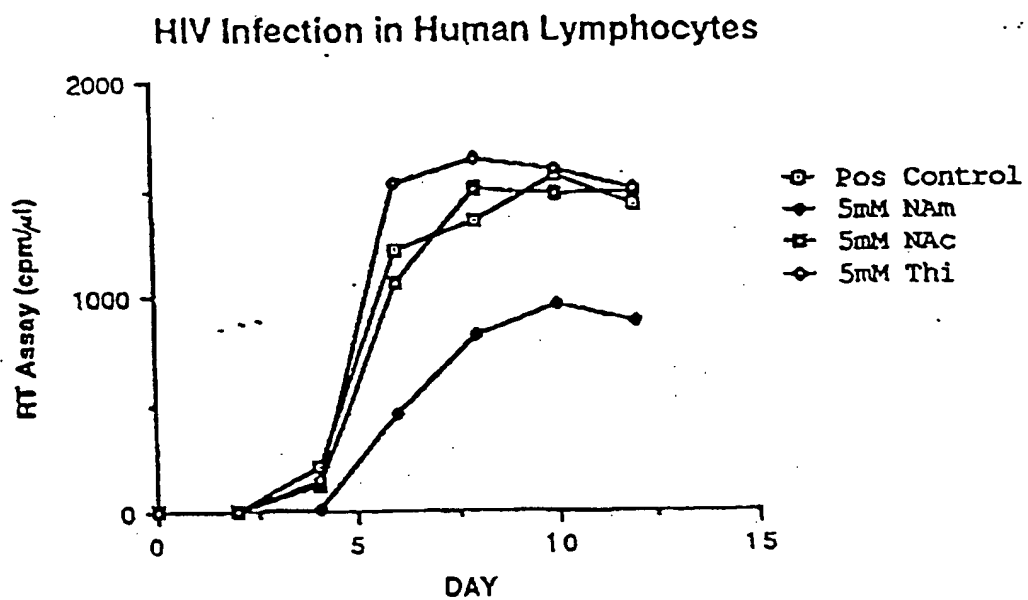


Figure 4

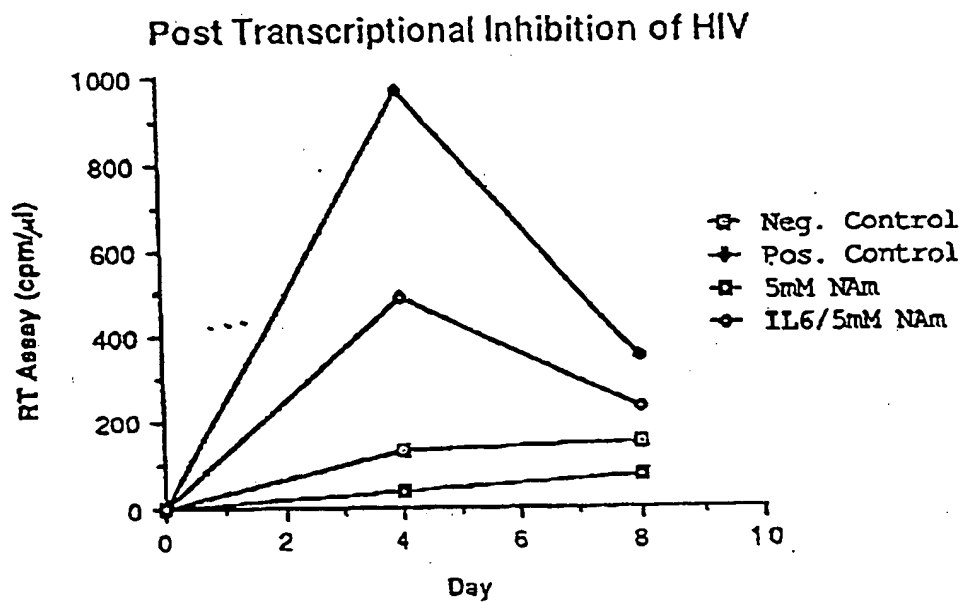
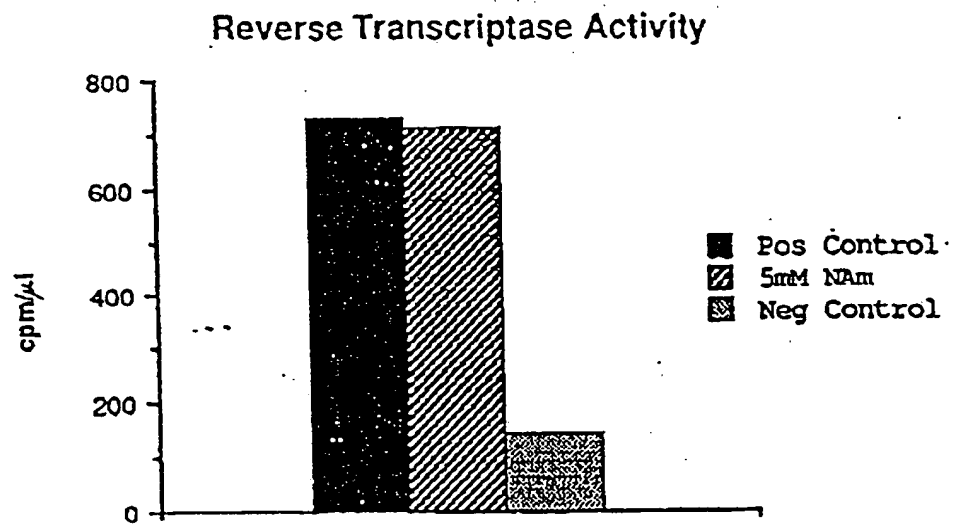


Figure 5



Micronutrient Profiles in HIV-1-Infected Heterosexual Adults

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Summary: There is compelling evidence that micronutrients can profoundly affect immunity. We surveyed vitamin supplement use and circulating concentrations of 22 nutrients and glutathione in 64 HIV-1 seropositive men and women and 33 seronegative controls participating in a study of heterosexual HIV-1 transmission. We assayed antioxidants (vitamins A, C, and E; total carotenes), vitamins B₆ and B₁₂, folate, thiamin, niacin, biotin, riboflavin, pantothenic acid, free and total choline and carnitine, biopterin, inositol, copper, zinc, selenium, and magnesium. HIV-infected patients had lower mean circulating concentrations of magnesium ($p < 0.0001$), total carotenes ($p = 0.009$), total choline ($p = 0.002$), and glutathione ($p = 0.045$), and higher concentrations of niacin ($p < 0.0001$) than controls. Fifty-nine percent of HIV+ patients had low concentrations of magnesium, compared with 9% of controls ($p < 0.0001$). These abnormal concentrations were unrelated to stage of disease. Participants who took vitamin supplements had consistently fewer low concentrations of antioxidants, across HIV infection status and disease stage strata ($p = 0.0006$). Nevertheless, 29% of the HIV+ patients taking supplemental vitamins had subnormal levels of one or more antioxidants. The frequent occurrence of abnormal micronutrient nutriture, as found in these HIV+ subjects, may contribute to disease pathogenesis. The low magnesium concentrations may be particularly relevant to HIV-related symptoms of fatigue, lethargy, and impaired mentation. **Key Words:** Micronutrient—HIV-1 infection—Trace metal—Immunity.

Deficiencies of single micronutrients are known to adversely affect the immune system by depression of cellular and humoral immunity and impairment of phagocytosis (1,2). Individuals infected

with the human immunodeficiency virus type 1 (HIV-1) may be particularly vulnerable to nutritional deficiencies that impair already compromised immune function. In a previous study of HIV-1 infected patients, we found that carotenes and ascorbate were below normal in 27% of the subjects, and vitamins E and A were low in 12% (3). Serum levels of micronutrients in HIV-1 patients have been associated with markers of immune function and stage of disease (4–7). Studies have shown that abnormalities in nutriture both accompany and predict HIV disease progression (6,8–10). These investigations assessed dietary intake or serum concentrations of one or a few micronutrients in selected cohorts. In

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The results of this study were presented in part at the Experimental Biology 94 meeting, Anaheim, California, April 24–28, 1994; and at the Keystone Symposium on HIV Pathogenesis, Keystone, Colorado, April 17–23, 1995.

Manuscript received July 11, 1995; accepted February 14, 1996.

this study, we surveyed concentrations of 22 circulating micronutrients and glutathione among 64 HIV-1 infected outpatients, ranging from asymptomatic to AIDS-diagnosed, and 33 seronegative controls. We also examined the association of self-directed vitamin supplementation on micronutrient status. Our data may provide insight for making clinical decisions and for understanding disease progression.

PATIENTS AND METHODS

Study Subjects

Between June 1992 and March 1993, HIV-1 infected and uninfected subjects were recruited from couples enrolled in a longitudinal study of heterosexual HIV-1 transmission conducted at the UMDNJ-New Jersey Medical School in Newark, New Jersey. Couples were referred by infectious diseases clinics, physicians, and New Jersey counseling and testing sites. Procedures are further described elsewhere (11). The same clinics and physicians referred additional HIV-1 infected patients, and additional seronegative controls were recruited from local staff. We obtained informed consent under a protocol approved by the New Jersey Medical School Institutional Review Board.

We determined HIV-1 serostatus by enzyme-linked immunosorbent assay and confirmed the status by Western blot. Participants provided information by structured interview on demographic background, clinical condition, weight, and on any medications, vitamins, and nutritional supplements they were currently taking. (The study protocol neither advised nor restricted the use of supplements.) Clinical stage of disease was assigned by the Centers for Disease Control 1993 Revised Classification System (12). For the seropositive subjects enrolled in the heterosexual transmission study, lymphocyte subpopulations were determined at 6-month intervals; for the remaining seropositive subjects, CD4⁺ and CD8⁺ counts were obtained from recent clinical records.

Blood Collection and Analysis

Blood for vitamin determinations was collected in lavender-top Vacutainers (Becton, Dickinson & Co., Rutherford, NJ, U.S.A.) with EDTA as an anticoagulant. Plasma for trace element analyses was collected in heparinized metal-free Vacutainers. Blood samples obtained at routine clinical follow-up were delivered to the laboratories ≤ 4 h of collection. Plasma concentrations of vitamins A, C, E, and carotenes were determined spectrophotometrically (13). Plasma concentrations of vitamin B₆ were analyzed with protozoa, and *Lactobacillus casei* was used for folate assays of plasma (13). Whole blood was analyzed with various protozoa for thiamin, biotin, nicotinates (niacin), pantothenates, vitamins B₆ and B₁₂, and riboflavin (13). Plasma carnitine, choline, biotin, and inositol determinations were carried out by previously described methods (14–17). Plasma concentrations of zinc, copper, and magnesium were measured by flame atomic absorption spectrophotometry (18,19); plasma selenium was measured by electrothermal atomic absorption (20).

Determination of glutathione required that subjects schedule a second blood draw at our laboratory, because the assay required immediate processing. Plasma was collected in metal-free Vacutainers containing EDTA and centrifuged, transferred to a polypropylene tube, mixed with 10% 5-sulfosalicylic acid, and centrifuged (21). Glutathione concentrations in the supernatant were determined by visible spectrophotometry at 412 nm (modified Beckman DB-GT). Vitamin and trace element assays were also repeated on the blood collected at this visit, to better characterize the micronutrient status of individuals.

Statistics

Measured concentrations were approximately normally distributed. To accommodate the correlation of duplicate determinations on the same subject, we estimated group mean concentrations by least square means and compared them by repeated measures analysis of variance (ANOVA), using SAS (SAS Institute Inc., Cary, NC, U.S.A.) software. Concentrations of all micronutrients except total choline were defined as *abnormal* if they were outside the laboratory's established normal range of mean ± 2 SDs for a reference population of ≥ 300 healthy adults. The normal range for total choline was based on the range of the seronegative controls. Group prevalences of out-of-range concentrations were based on determinations at the first visit and compared using StatXact@ (Cytel Software Corp., Cambridge, MA, U.S.A.) exact contingency table analyses. The subject was the unit of analysis. Associations between micronutrient concentrations and lymphocyte counts were described by Pearson correlation coefficients. We performed stepwise multiple regression to evaluate the joint effects of micronutrients on lymphocyte counts; final models included correlates after backward elimination at $p \geq 0.10$. All p values are two-tailed; $p < 0.05$ is considered statistically significant.

RESULTS

We obtained micronutrient data from 97 study subjects. Sixty-nine subjects (52 seropositive, 17 seronegative controls) provided blood samples at two visits, for a total of 166 micronutrient determinations. The interval between visits ranged from 28 to 126 days (median 49 days).

Subject characteristics are presented in Table 1, by infection status and stage of disease. Sixty-four (65%) subjects were seropositive for HIV-1 infection: 19 were diagnosed with AIDS, 18 had HIV-related symptoms, and 27 remained asymptomatic. Forty-three HIV-positive subjects (67%) and 17 seronegative controls (52%) were male. No female was pregnant. Sixty percent of the subjects were white non-Hispanic (33 HIV-positive, 25 controls); 26% were Hispanic (21 HIV-positive, 5 controls); 13% were black non-Hispanic (10 HIV-positive, 2 controls); and 1 control was Asian. Seronegative controls were more predominantly white (76 vs. 52%); approximately two-thirds of nonwhite sub-

TABLE 1. Characteristics of subjects at first visit

Diagnostic category	N	Male (%)	Age (yr) Mean \pm SEM	CD4 ⁺ in cells/ml Mean \pm SEM	Percent taking vitamin supplements
HIV seronegative	33	52	40 \pm 1.3		42
HIV seropositive CDC stage ^a					
A (asymptomatic)	27	67	37 \pm 1.5	508 \pm 51	63
B (symptomatic)	18	61	36 \pm 2.0	208 \pm 35	73
C (AIDS)	19	74	37 \pm 1.6	222 \pm 54	63

CDC, Centers for Disease Control.

jects in both groups were Hispanic. Controls were slightly but not significantly older: mean 40 versus 36 years. Twenty-five male and three female seropositive subjects were i.v. drug users: three men were bisexual. Fifteen men and 18 women attributed their infection to heterosexual transmission.

HIV-infected subjects reported taking antiviral medication at the time of 82 (71%) of their 116 visits. Concurrent prophylaxis for *Pneumocystis carinii* pneumonia was infrequently reported (at 16 visits). Nine patients (14%) reported a weight loss of ≥ 5 kg in the past 6 months (maximum: 12 kg), and none showed clinical evidence of wasting (weight loss $> 10\%$ in 6 months, with recurrent fever or diarrhea). All HIV-infected subjects were clinically stable outpatients.

Intake of Supplemental Vitamins

Current consumption of multivitamin supplements was reported at 98 of the 166 subject visits (59%). Supplemental consumption of vitamin C was reported at 40 visits (24%), of vitamin E at 32 (19%), and of B-complex at 23 (14%). These vitamins were taken in various combinations, typically in addition to a multivitamin supplement. Ten subjects reported taking vitamin supplements at only one of their two visits (5 at the first, 5 at the second). Four subjects took supplements other than vitamins (1: Ca, Mg; 1: protein, iron; 2: phosphorus). HIV-infected subjects reported taking some vitamin supplement at 66% of their 116 study visits, compared to 46% of 50 visits by controls.

HIV Infection Status and Micronutrient Levels

Mean Micronutrient Levels

Mean plasma concentrations of micronutrients are presented by serostatus in Table 2. Concentra-

tions of antioxidants (vitamins A, C, E, total carotenenes, and glutathione) tended to be lower among those with HIV infection than among seronegative controls: the difference was significant in repeated measures ANOVA only for carotenenes ($p = 0.009$) and glutathione ($p = 0.045$). Levels of the vitamin B complex and related metabolites were comparable for HIV-infected and uninfected subjects, except that niacin was significantly higher among HIV-infected patients ($p < 0.0001$) and total choline was lower ($p < 0.002$). Concentrations of magnesium were also lower in HIV-infected subjects ($p < 0.0001$), but levels of zinc, selenium, and copper did not differ between infected and uninfected subjects.

In analyses of HIV-infected subjects classified by stage of illness, mean concentrations of micronutrients did not vary systematically with increasing severity of illness.

Abnormal Micronutrient Levels

Table 2 presents the proportion of micronutrient concentrations that fell below and above the normal laboratory range. These frequencies are based on 166 determinations that included replicates for subjects with two visits. The frequencies based solely on the first visit are similar and were used for hypothesis testing of group differences.

At the first visit, significantly more HIV-1 infected subjects had low concentrations of magnesium (59 vs. 9%, $p < 0.0001$) and carotenenes (25 vs. 3%, $p = 0.009$) than uninfected controls. Low concentrations of vitamin C (20%) and folic acid (15%) were not more prevalent among HIV-infected patients than among controls.

We investigated the possibility that low magnesium concentrations among the HIV-infected cohort reflected a greater number of heavy drinkers (≥ 3 drinks a day). HIV infection remained a significant predictor of low magnesium but heavy drinking was not, when the factors were considered

TABLE 2. Circulating micronutrient concentrations in HIV-infected and seronegative subjects^a

	Normal range	Unit	Percentage beyond normal range				Least squares mean \pm SE		p Value ^a
			Below range (%)		Above range (%)		HIV +	HIV -	
			HIV +	HIV -	HIV +	HIV -			
Vitamin A	0.87-2.62	$\mu\text{mol/L}$	0	0	23	35 ^c	2.13 \pm 0.10	2.41 \pm 0.15	0.10
Vitamin C	23-85	$\mu\text{mol/L}$	20	10	7	8	44.9 \pm 3.1	48.8 \pm 4.8	
Vitamin E	14-35	$\mu\text{mol/L}$	4	0	21	26	29.3 \pm 1.7	33.9 \pm 2.6	0.11
Total carotenes	1.5-5.6	$\mu\text{mol/L}$	26	2 ^d	3	4	2.40 \pm 0.20	3.33 \pm 0.32	
Glutathione ^f							4.71 \pm 0.27	5.58 \pm 0.33	0.009
Vitamin B ₁₂	200-1390	pmol/L	2	0	3	2	573 \pm 40	450 \pm 64	0.045
Folic acid	11-54	nmol/L	15	16	25	18	38.5 \pm 4.3	34.0 \pm 6.8	
Vitamin B ₆	179-479	nmol/L	2	0	18	8	389 \pm 42	317 \pm 67	0.08
Thiamin	0.07-0.21	$\mu\text{mol/d}$	0	0	5	4	0.163 \pm .004	0.148 \pm .007	
Niacin	28-57	$\mu\text{mol/L}$	4	8	9	0 ^c	43.9 \pm 0.89	37.4 \pm 1.38	0.08
Biotin	820-3070	pmol/L	0	0	25	29	2840 \pm 143	2820 \pm 94	0.0001
Riboflavin	265-1330	nmol/L	0	0	9	8	954 \pm 27	988 \pm 43	
Pantothenic acid	0.91-4.56	$\mu\text{mol/L}$	0	0	4	4	2.28 \pm 0.12	1.96 \pm 0.20	0.15
Free choline	3.2-6.2	$\mu\text{g/ml}$	0	0	17	7 ^c	4.9 \pm 0.11	4.8 \pm 0.18	
Total choline	170-665	$\mu\text{g/ml}$	4	2	1	2	300 \pm 11.6	364 \pm 18.4	0.002
Biopterin	3.0-7.2	nmol/L	0	2	28	26	6.3 \pm 0.21	5.9 \pm 0.38	
Inositol	17-67	$\mu\text{mol/L}$	4	2	1	0	29.4 \pm 1.0	32.2 \pm 1.6	0.11
Free carnitine	29-49	$\mu\text{mol/L}$	0	4	37	48	47.8 \pm 1.4	49.6 \pm 2.2	
Total carnitine	33-61	$\mu\text{mol/L}$	0	2	35	38	58.3 \pm 1.6	55.8 \pm 2.5	
Copper	10.2-22.8	$\mu\text{mol/L}$	0	2	0	8 ^c	16.4 \pm 0.38	16.7 \pm 0.58	
Zinc	10.7-18.4	$\mu\text{mol/L}$	4	0	7	8	15.0 \pm 0.34	15.1 \pm 0.52	
Selenium	0.89-2.03	$\mu\text{mol/L}$	2	0	18	16	1.72 \pm 0.04	1.71 \pm 0.06	
Magnesium	0.74-1.23	mmol/L	52	12 ^c	0	0	0.74 \pm .007	0.79 \pm .011	0.0001

^a Sample size is $n = 46$ to 50 values for HIV - subjects and $n = 112$ to 116 values for HIV + subjects, except for glutathione ($n = 17$ HIV - subjects, $n = 35$ HIV + subjects). Superscripts c-e indicate significant difference between HIV + and HIV - prevalence of out-of-range levels at first visit, by Fisher's exact test.

^b Significance level of repeated measures ANOVA test for differences in means of HIV - and HIV - subjects.

^c $0.03 < p < 0.05$.

^d $p = 0.009$.

^e $p < 0.0001$.

^f Normal range not established for glutathione.

jointly in a logistic regression (respective p values: 0.0001, 0.14). Neither weight loss nor diarrhea in the preceding 6 months was related to the low serum magnesium among HIV-infected patients.

Micronutrient concentrations above normal ranges were observed in both seronegative controls and HIV-infected subjects (Table 2). At the first visit, more controls than HIV-infected subjects had high levels of vitamin A (38 vs. 17%, $p = 0.043$) and high copper levels (9 vs. 0%, $p = 0.038$). More HIV-infected subjects had high free choline levels than controls (30 vs. 10%, $p = 0.036$), and more had high niacin levels than controls (13 vs. 0%, $p = 0.049$). Current use of antiviral medication by HIV-infected subjects did not appear related to the frequency of high concentrations of folic acid.

Use of Vitamin Supplements as a Factor in Serum Concentrations

Vitamin supplementation was defined as reported use of multivitamins or any specified vitamins. The

relationship between intake of supplements and plasma concentrations is presented for data from the first visit. Results based on second visit data were similar.

HIV-infected subjects who were taking vitamin supplements had significantly higher mean levels of B₁₂, folic acid, B₆, thiamin, pantothenic acid, and vitamin C. We observed no differences by stage of illness. The only significant difference among controls was that those taking vitamins had higher concentrations of vitamin C ($p = 0.01$) than those not taking vitamins.

Because supplementation was more common among HIV-1 infected subjects than controls, comparisons of mean micronutrient levels were adjusted for supplemental vitamin intake. The findings were modified only slightly: The difference between vitamin E concentrations of HIV-infected subjects and controls attained significance ($p = 0.03$), while the difference in carotene levels was borderline ($p = 0.06$).

We also examined the impact of vitamin supplementation on the frequency of abnormal micronutrient levels. In analyses that adjusted for vitamin supplementation, HIV-infected subjects had significantly more high niacin levels, more low carotene levels, and fewer high levels of vitamin A than controls. After adjustment for vitamin supplementation, the prevalences of low- and high folate concentrations were no different among HIV-infected patients and controls.

Prevalence of Low Blood Antioxidant Levels at First Visit

The number of subjects with below-normal blood concentrations of antioxidants (vitamins A, C, E, and carotenes) at the first visit is shown in Table 3, by infection status and stage-of-illness category (seronegative, asymptomatic, symptomatic, AIDS). Eight patients had two low concentrations, only one of whom was taking vitamins. No subject had more than two low antioxidant concentrations, and none had a low vitamin A concentration. Ten of 19 HIV-infected subjects (53%) who were not taking vitamins had at least one low concentration of vitamins C and E or carotenes. Low antioxidant concentrations were more frequent in later disease stages (22% of asymptomatic patients and 46% of symptomatic and AIDS-diagnosed patients). Within each HIV disease category, those taking vitamin supplements tended to have fewer low antioxidant con-

centrations than those not taking vitamins. Across all four infection and stage-of-disease strata, supplemental intake was a highly significant factor for having fewer low antioxidant concentrations (stratified exact trend test, $p = 0.0006$). Nevertheless, 29% (13 of 45) of HIV-infected patients had low concentrations of at least one antioxidant despite vitamin supplementation.

Glutathione: Correlation with Antioxidant and Magnesium Levels

Plasma glutathione concentrations were positively correlated with concentrations of vitamin C ($r = 0.33$, $p = 0.013$) but were not correlated with vitamins A and E or carotenes. In a stepwise regression of glutathione on these antioxidants, vitamin C was the only significant correlate ($p = 0.0026$). Glutathione and magnesium concentrations were also positively correlated ($r = 0.29$, $p = 0.03$).

Correlation of CD4s and CD4/CD8 Ratios with Micronutrient Levels

For HIV-1 infected subjects, lymphocyte counts closest in time to micronutrient determinations were correlated with the micronutrient concentrations (median interval between lymphocyte and micronutrient assays = 28 days; 90th percentile <90 days). In univariate analyses, higher niacin levels were associated with lower CD4 counts ($r = 0.43$, p

TABLE 3. Prevalence at first visit of low blood antioxidant concentrations by infection status and vitamin intake*

Clinical category	Taking vitamins	Number (%) of subjects			Total
		Number of low plasma antioxidant concentrations			
		None	One	Two	
Seronegative	No	16 (84)	3 (16)	0 (0)	19
	Yes	12 (92)	1 (8)	0 (0)	13
Seropositive	No	9 (47)	3 (16)	7 (37)	19
	Yes	32 (71)	12 (27)	1 (2)	45
A (asymptomatic)	No	7 (70)	2 (20)	1 (10)	10
	Yes	14 (82)	3 (18)	0 (0)	17
B (symptomatic)	No	1 (20)	1 (20)	3 (60)	5
	Yes	9 (69)	3 (23)	1 (8)	13
C (AIDS diagnosed)	No	1 (25)	0 (0)	3 (75)	4
	Yes	9 (60)	6 (40)	0 (0)	15
All subjects	No	25 (66)	6 (16)	7 (18)	38
	Yes	44 (76)	13 (22)	1 (2)	58
Total		69 (72)	19 (20)	8 (8)	96

* Antioxidants included vitamins A, C, and E and total carotenes. Maximum possible number of low antioxidant concentrations = 4.

= 0.0004), and higher bipterin levels were weakly associated with lower CD4 counts ($r = 0.25$, $p = 0.05$). Niacin levels showed a corresponding association with the numeric ratios of CD4 to CD8 counts ($r = -0.48$, $p = 0.0003$). In addition, CD4/CD8 ratios were positively correlated with carotenes ($r = 0.32$, $p = 0.02$) and riboflavin ($r = 0.29$, $p = 0.04$), and weakly related to vitamin B₆ ($r = 0.24$, $p = 0.07$). Significance levels for all other correlations were >0.15 .

Table 4 presents the results of multiple regression to select the best joint predictors of CD4 counts and CD4/CD8 ratios among blood micronutrients and trace metals. Niacin and bipterin remained significant inverse correlates of CD4⁺ counts, and riboflavin was a significant positive correlate. In the best model for CD8 counts, carotenes were a weak negative correlate and vitamins E and B₁₂ were weak positive correlates, although jointly these factors explained only 20% of the variation in CD8 (model not shown). Lower CD4/CD8 ratios thus were associated with higher niacin levels and lower riboflavin levels because of lowered CD4 counts; on the other hand, lower CD4/CD8 ratios were associated with lower carotene levels and higher vitamin E and B₁₂ levels because of higher CD8 counts.

DISCUSSION

This comprehensive study presents a cross-sectional profile of micronutrient status in a cohort

TABLE 4. Multiple regression models of CD4⁺ counts and CD4/CD8 ratios on micronutrient*

Dependent variable	Predictor	Parameter estimate	p
CD4 ⁺	Intercept	1308	0.0001
	Niacin (μg/ml)	-105	0.0006
	Biopterin (ng/ml)	-220	0.0003
	Riboflavin (ng/ml)	1.12	0.0073
	Biotin (μg/ml)	-0.25	0.058
	Total carnitine (μg/ml)	-26.6	0.051
	Vitamin B ₁₂ (ng/ml)	-0.24	0.093
CD4/CD8 ratio	Intercept	0.73	0.0001
	Niacin (μg/ml)	-0.102	0.0002
	Carotenes (μg/dl)	0.0018	0.0002
	Vitamin B ₁₂ (ng/ml)	-0.0007	0.0012
	Vitamin E (mg/dl)	-0.25	0.0017
	Riboflavin (ng/ml)	0.0010	0.017
	Total choline (μg/ml)	0.00090	0.015
	Vitamin A (μg/ml)	-0.0033	0.016

* Stepwise selection on 22 micronutrients, terminated by backward elimination when $p \geq 0.10$. For CD4⁺, $N = 59$. Model $R^2 = 0.39$; for CD4/CD8 ratio, $N = 46$; Model $R^2 = 0.60$.

of HIV-infected outpatients and seronegative controls. The cohort includes men and women, spans the three CDC categories of illness, and includes subjects infected by i.v. drug use and homosexual and heterosexual transmission. Subjects' diet and vitamin use were unrestricted. Although the study was not population-based, the observations may be more generalizable to the free-living HIV-positive population than studies that attempt to control intake or that focus on late-stage cases after onset of wasting.

Of the vitamin-mineral concentrations found to be above or below the laboratory normal range and accompanied by consistent and statistically significant differences in mean concentrations between HIV-positive and HIV-negative subjects, five findings stand out: HIV-positives have lower total carotene, glutathione, total choline, and magnesium concentrations, but higher niacin concentrations.

A substantial percentage of HIV⁺ subjects had low plasma antioxidant levels, in particular, low vitamin C and total carotene concentrations. A striking finding was that self-supplementation appears to spare many HIV⁺ subjects from low antioxidant concentrations at all stages of disease. However, 29% of those taking supplements still presented with one or more low antioxidant levels.

These results confirm other investigations (1,3,8) that showed low concentrations of vitamin C, folate, and carotenes among HIV-infected subjects. No below-normal concentrations of vitamin A were observed; this is not surprising since homeostatic regulation prevents low serum retinol concentrations unless there is frank deficiency (22). There is evidence that vitamin A helps to maintain the integrity of mucosal surfaces and that maternal vitamin A deficiency contributes to vertical transmission of HIV-1 (23). That low vitamin A concentrations were not seen could partially explain the low incidence of heterosexual transmission among these HIV-discordant couples.

The reduced glutathione concentrations found among HIV-infected subjects are similar to the results of other studies and are potentially important because glutathione is a cellular antioxidant (24). The significant association between plasma concentrations of ascorbate and glutathione is consistent with a report demonstrating that vitamin C consumption can increase red blood cell glutathione concentrations (25). However, plasma glutathione concentrations may not correlate with red blood cell or tissue glutathione levels. It is also not clear

that increasing dietary glutathione will increase plasma or cellular glutathione or glutathione peroxidase concentrations. Administration of the glutathione precursor *N*-acetylcysteine to HIV-infected individuals is being investigated by other researchers.

Niacin levels were higher among HIV-infected subjects, both on average and in the proportion with above-normal levels. Furthermore, higher niacin levels were highly correlated with lower CD4⁺ counts. The significance of this inverse relationship is not clear. We have not found other reports that document this association or that would help explain this finding.

A 20% prevalence of low serum magnesium concentrations among 224 HIV-1 infected patients was recently reported (26), along with a 46% prevalence of low erythrocyte magnesium concentrations. As in our study, CD4⁺ lymphocyte counts were not correlated with serum magnesium levels, although they did correlate with red blood cell levels (26). However, another study of 31 AIDS patients observed no evidence of abnormal serum magnesium levels (27). Isolated cases of magnesium deficiency in AIDS patients have been attributed to pentamidine therapy (28). Neither antiviral nor prophylactic medications explain the low magnesium levels observed in our patients nor were their low magnesium levels explained by a history of heavy drinking, wasting syndrome, or recent diarrhea.

Magnesium deficiency may affect both antibody synthesis and T cell function (29). In induced magnesium deficiency in rats and hamsters, cardiac myopathic lesions appear rapidly. These cardiac abnormalities are accompanied by a striking increase in production of the macrophage-induced cytokines interleukin-1, interleukin-6, and tissue necrosis factor alpha (30). The number and severity of the cardiac lesions can be reduced by vitamin E administration, suggesting the possible role of free radicals, perhaps resulting from a cytokine-induced inflammatory response (31). Magnesium deficiency in rats also reduces production of glutathione (32). In the present study, there was a weak but significant linear correlation ($r = 0.29$) between low plasma glutathione and magnesium levels.

The multiple signs and symptoms of magnesium deficiency include lethargy, weakness, fatigue, and decreased mentation (33); these are common complaints of HIV-infected patients. We measured plasma concentrations of magnesium, a predominately intracellular cation. Future studies should

examine cellular concentrations in lymphocytes and red blood cells, because lower plasma magnesium may reflect a redistribution of magnesium from the extracellular fluid to the intracellular compartment in HIV-infected cells.

There are two additional points about the magnesium findings. First, although about half of our HIV-positive subjects had low plasma magnesium concentrations, the difference between the means of the HIV-positives and HIV negatives was only 0.05 mmol/L. Whether this difference is biologically significant is unclear. Second, our lower limit of normal for plasma magnesium is 0.74 mmol/L. That lower limit is similar to many laboratories, but in others the lower limit is as low as 0.65 mmol/L. If we had used the lower value, all but five of our HIV-positive individuals would have been considered "normal." Thus, HIV-positive persons may have relative deficits of magnesium rather than frank deficiency.

The normal zinc and copper concentrations in these HIV-infected subjects may reflect absence of acute-phase infections at the time of blood sampling, because infection can increase serum copper and decrease serum zinc (34). Other reports of low zinc and high copper levels among HIV-positive patients have noted that levels were marginally abnormal (35,36) or that abnormal levels were also prevalent among controls (37). The finding of normal selenium concentrations is consistent with another study (38). Thus, our results are not at variance with results of other studies.

Clinical stage of illness had little apparent effect on the micronutrient status of the HIV-infected subjects. However, all of our subjects, even those already diagnosed with AIDS, were in relatively good health and functioning well as outpatients, with no clinical evidence that nutrient metabolism was compromised by wasting or severe diarrhea. The sample sizes were not large enough to detect differences in micronutrient concentrations between stage B (symptomatic) and C (AIDS-diagnosed) subjects that might well be modest in a currently healthy population.

High concentrations of vitamin E, total carotenes, and folates were often observed, possibly due to vitamin supplementation by a majority of the subjects. The inverse correlation of bipterin with CD4 counts (Table 4) could be explained by disease progression, which is marked by elevated levels of neopterins (39), a molecule similar to bipterin. High levels of bipterins are also seen during progression

and immunotherapy of various cancers (40). The lower mean total choline concentrations along with normal free choline could reflect inability of HIV-infected patients to convert free choline to lecithin (phosphatidylcholine), an essential component of cell membranes (41). Reduced total choline concentrations in seropositive subjects could contribute to T cell dysfunction (1).

Conclusions about the benefits of multivitamin supplements are limited by the observational design and lack of controlled intervention. We did not ask subjects for information on diet or for dosages contained in their multivitamin or single vitamin supplements. It was thus not possible to quantify intake of micronutrients or to assess the relative contributions of diet and supplementation.

Seronegative controls were not matched demographically to HIV-seropositive subjects. However, they were similar in age and gender. HIV-discordant couples from the transmission study were necessarily opposite in gender, but they were typically of the same race and comparable age, lived together, and presumably shared a similar diet. Controls enrolled from local staff included some, albeit fewer, nonwhite subjects (20%). Thus, ethnic differences between HIV-positives and controls were unlikely to account for observed differences in micronutrients.

Our results provide additional evidence that micronutrient status may be compromised in HIV-infected subjects in the early stages of disease but may be at least partially corrected among some HIV-1 patients by the use of micronutrient supplements. Controlled interventions are needed to determine whether nutritional supplementation can remedy the possible consequences of abnormal nutritional status. It is possible that prolongation of the interval between infection and symptoms observed over the last decade might relate in part to better nutrition or the use of nutritional supplements. That possibility should encourage additional studies of nutritional status and therapy, of the effects of relatively high doses of supplements (in particular, antioxidants), and of the role of glutathione and its precursors. The role of magnesium deficits in HIV infection and the significance of higher niacin and lower total choline levels also merit further investigation.

Acknowledgment: The Heterosexual HIV Transmission Study was supported by National Institutes of Health Contract NO1-A1-95013, from the National Institute of

Allergy and Infectious Diseases. We thank Michael Jaffee for computer programming assistance.

REFERENCES

1. Biesel WR. Single nutrients and immunity. *Am J Clin Nutr* 1982;35:417-68.
2. Klurfeld DM. *Nutrition and immunology*. New York: Plenum Press, 1993.
3. Bogden JD, Baker H, Frank O, et al. Micronutrient status and human immunodeficiency virus (HIV) infection. *Ann NY Acad Sci* 1990;587:189-95.
4. Baum MK, Shor-Posner G, Bonvehi P, et al. Influence of HIV infection on vitamin status and requirements. *Ann NY Acad Sci* 1992;669:165-73.
5. Baum MK, Mantero-Atienza E, Shor-Posner G, et al. Association of vitamin B₆ status with parameters of immune function in early HIV-1 infection. *J Acquir Immune Defic Syndr Hum Retrovirol* 1991;4:1122-32.
6. Semba RD, Graham NMH, CIAffa WT, Margolick JB, Clement L, Vlahov D. Increased mortality associated with Vitamin A deficiency during human immunodeficiency virus type 1 infection. *Arch Intern Med* 1993;153:2149-54.
7. Fordyce-Baum MK, Mantero-Atienza E, Morgan R, van Riel F, Beach RS. Toxic levels of dietary supplementation in HIV-1 infected patients. *Arch AIDS Res* 1990;4:149-57.
8. Coodley GO, Coodley MK, Nelson HD, Loveless MO. Micronutrient concentrations in the HIV wasting syndrome. *AIDS* 1993;7:1595-1600.
9. Tang AM, Graham NMH, Kirby AJ, McCall LD, Willett WC, Saah AJ. Dietary micronutrient intake and risk of progression to acquired immunodeficiency syndrome (AIDS) in human immunodeficiency virus type 1 (HIV-1)-infected homosexual men. *Am J Epidemiol* 1993;138:937-51.
10. Abrams B, Duncan D, Hertz-Picciotto I. A prospective study of dietary intake and acquired immune deficiency syndrome in HIV-seropositive homosexual men. *J Acquir Immune Defic Syndr Hum Retrovirol* 1993;6:949-58.
11. Kennedy CA, Skurnick J, Wan JY, et al. Psychological distress, drug and alcohol use as correlates of condom use in HIV-serodiscordant heterosexual couples. *AIDS* 1993;7:1493-9.
12. 1993 revised classification system for HIV infection and expanded surveillance case definition for AIDS among adolescents and adults. *MMWR* 1992;41:1-19.
13. Baker H, Frank O. *Clinical vitaminology: methods and interpretation*. New York: Interscience, 1968.
14. Baker H, DeAngelis B, Baker ER, Reddi A, Khalil M, Frank O. Routine microbiological assay for carnitine activity in biological fluids and tissues. *Food Chemistry Analytical Methods Section* 1992;43:141-6.
15. Baker H, Frank O, Tuma DJ, Barak AJ, Sorrell MF, Hutner SH. Assay for free and total choline activity in biological fluids and tissues of rats and man with *torulopsis pintolopessi*. *Am J Clin Nutr* 1978;31:532-40.
16. Baker H, Frank O, Bacchi C, Hutner S. Biopterin content of human and rat fluids and tissues determined protozoologically. *Am J Clin Nutr* 1974;27:1247-53.
17. Baker H, DeAngelis B, Baker ER, Khalil M, Reddi AS, Frank O. Routine microbiological assay for myoinositol for clinical use. *J Micronutrient Analysis* 1990;8:223-32.
18. Bogden JD, Thind IS, Kemp FW, Caterini H. Plasma concentrations of calcium, chromium, copper, iron, magnesium, and zinc in maternal and cord blood and their relationship to low birth weight. *J Lab Clin Med* 1978;92:455-62.
19. Bogden JD, Troiano RA. Plasma calcium, copper, magnesium, and zinc concentrations in patients with the alcohol withdrawal syndrome. *Clin Chem* 1978;24:1553-6.

20. Jacobson BE, Lockitch G. Direct determination of selenium in serum by graphite-furnace atomic absorption spectrometry with deuterium background correction and a reduced palladium modifier: age-specific reference ranges. *Clin Chem* 1988;34:709-14.
21. Griffith OW. Determination of glutathione and glutathione disulfide using glutathione reductase and 2-vinylpyridine. *Anal Biochem* 1980;106:207-12.
22. Olsen JA. The irresistible fascination of carotenoids and vitamin A. *Am J Clin Nutr* 1992;57:833-9.
23. Semba RA, Miotti PG, Chipangwi JD, et al. Maternal vitamin A deficiency and mother-to-child transmission of HIV-1. *Lancet* 1994;343:1593-7.
24. Buhl R, Jaffe HA, Holroyd KJ, et al. Systemic glutathione deficiency in symptom-free HIV-positive individuals. *Lancet* 1989;2:1294-7.
25. Johnston CS, Meyer CG, Sriakshmi JC. Vitamin C elevates red blood cell glutathione in healthy adults. *Am J Clin Nutr* 1993;58:103-5.
26. Berger DS, Reiter VM, Vorce DE, et al. Prevalence of red blood cell magnesium deficiency in HIV-1 infected patients and its association with fatigue and myalgia. *J Am Coll Nutr* 1994;13:522.
27. Heise W, Nehm K, L'Age M. Concentrations of magnesium, zinc, and copper in serum of patients with acquired immunodeficiency syndrome. *J Clin Chem Clin Biochem* 1989;27:515-17.
28. Gradon JD, Fricchione L, Sepkowitz D. Severe hypomagnesemia associated with pentamidine therapy. *Rev Infect Dis* 1991;13:511-12.
29. Kubene KS. Role of magnesium in immunity. *J Nutr Immunol* 1993;2:107-26.
30. Weglicki WB, Phillips TM, Freedman AM, Cassidy MM, Dickens BE. Magnesium deficiency elevates circulating levels of inflammatory cytokines and endothelin. *Mol Cell Biochem* 1992;110:169-73.
31. Freedman AM, Atrakahd AH, Cassidy MM, Weglicki WB. Magnesium induces cardiomyopathy, protection by vitamin E. *Biochem Biophys Res Comm* 1990;170:1102-6.
32. Mills BJ, Landeman RD, Lang CA. Magnesium deficiency inhibits biosynthesis of blood glutathione and tumor growth in the rat. *Proc Soc Exp Biol Med* 1986;181:326-32.
33. Shils ME. Magnesium. In: Braun ML, ed. *Present knowledge in nutrition*, 6th ed. Washington: International Life Sciences Institute, 1990:224-32.
34. Bogden JD, Lintz DI, Joselow MM, Charles J, Salaki JS. Copper:zinc ratios in plasma, whole blood, and erythrocytes in pulmonary tuberculosis. *Health Lab Sci* 1978;15:38-43.
35. Falutz J, Tsoukas C, Gold P. Zinc as a co-factor in human immunodeficiency virus-induced immunosuppression. *JAMA* 1988;259:2850-1.
36. Beach RS, Mantero-Atienza E, Shor-Posner G, et al. Specific nutrient abnormalities in asymptomatic HIV-1 infection. *AIDS* 1992;6:701-8.
37. Graham NM. On 'Specific nutrient abnormalities in HIV-1 infection' [Letter]. *AIDS* 1992;6:1552-3.
38. Graham NMH, Sorensen D, Odaka N, et al. Relationship of serum copper and zinc levels to HIV-1 seropositivity and progression to AIDS. *J Acquir Immune Defic Syndr Hum Retrovirol* 1991;4:976-80.
39. Jacob RA, Kelley DS, Pianalto PS, et al. Immunocompetence and oxidant defense during ascorbate depletion of healthy men. *Am J Clin Nutr* 1991;54:1302-9.
40. Baker H, Marcus SL, Frank O, et al. Interleukin-2 enhances bipterins and catecholamines production during adoptive immunotherapy for various cancers. *Cancer* 1989;64:1226-31.
41. Zeisel SH. Choline deficiency. *J Nutr Biochem* 1990;1:332-49.

Example of Nicotinamide's Failure to Act as an Antiviral In Vivo

Patient #1

Time	Viral Load
pretreatment	> 1,000,000 copies
1 week on therapy	765,000 copies
1 month on therapy	>1,000,000 copies

Explanation – this is the extent of available viral load data from the study which lead to the '552 patent application examples. This patient took 3 grams of nicotinamide per day for 2 months. The viral load is a measure of the amount of virus in 1 ml of patient blood. A patient on effective antiviral therapy will have a steady decrease in their viral load until it becomes undetectable [i.e. less than 50 copies per ml of blood].

Conclusion – while this is not conclusive scientific data, this early data from patient #1 lead to the impression that nicotinamide did not have a clear in vivo antiviral effect. While the one week result looked somewhat favorable, when viewed as a complete set the variations in the three time points are within the daily variations seen in this test in people not on antiviral therapy. Given the emerging data I was developing at this time on increases in plasma tryptophan, the focus of the study became the tryptophan results and further viral load measurements were not obtained on this patient or the other three patients in the patent examples.

In re Application of: Michael F. Murray

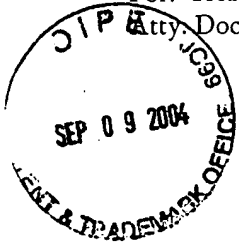
Art Group: 1017

Serial No.: 09/609,552 Examiner: Russell Travers, J.D., PhD

Filed: 6/30/2000

For: Treatment of Retrovirus Induced Derangements with Niacin Compounds

Atty: Docket No.: PHJM0609-001



DECLARATION OF DAVID G. SIDEBOTTOM, M.D.

I hereby declare that:

1. I am a licensed specialist physician in the field of Infectious Diseases in the state of Massachusetts.
2. I have been practicing Infectious Diseases as a clinical specialty for eighteen years.
3. I treated patients with retroviruses on a regular basis since 1986.
4. I have no interest whatsoever in the outcome of this matter. I was a professional colleague of Dr. Murray at both The Holy Family Hospital and Lawrence General Hospital when he had practiced full-time clinical Infectious Diseases. Dr. Murray asked me to review his patent application and comment on whether I could practice his invention based on his patent application.
5. This declaration reflects my independent professional opinion.

Knowledge of State of Art in June of 2000

6. I am familiar with the types of treatment available to patients infected with retroviruses in June of 2000.

Ability to practice the claimed invention using the application as a guide.

7. I have reviewed the patent application titled "Treatment of Retrovirus Induced Derangements with Niacin Compounds" filed on behalf of Michael F. Murray, M.D. on June 30, 2000. A true and correct copy of the patent application I reviewed is attached hereto as Exhibit A.
8. Based upon my review, I could practice the invention using the application as a guide for the following reasons:
 - a. The application provides information on the administration of niacin and well as cites references for the reader to learn more information about the administration and effects of niacin.

- b. In addition, medical doctors or those in the medical field are often familiar with niacin. Those who are not readily familiar with niacin know that much information can be found regarding the administration and effects of niacin on "Medline", in journals, books and other commonly available resources.
 - c. The application tells me that the preferred method to combat plasma tryptophan depletion is to "administer niacin in 'pharmacological doses'". (Application, pg. 7, line 12).
 - d. The application recommends that I administer a dose greater 20 milligrams per day because a lesser dose would not be expected to produce the pharmacological effect of combating plasma tryptophan depletion. (Application, pg 8, line 1).
 - e. The application informs me to expect pharmacological activity to occur at a dose of 100 milligrams per day. (Application, pg. 8, lines 10-11).
 - f. The application informs me to expect that a patient will under go a reversal of systemic tryptophan depletion upon the daily administration of 100 milligrams of niacin. (Application, pg. 8, lines 10-15).
 - g. The application informs me that the preferred method of administration of niacin in this invention is oral administration. (Application, pg 9, lines 2-3).
 - h. The application informs me that the preferred dose is 500 milligrams of niacin per day. (Application, pg. 9, lines 3-4).
 - i. The application informs me that the preferred form of niacin to practice this invention is nicotinamide. (Application, pg. 9, lines 3-4).
 - j. By way of example, the application informs me that administering 3 grams of nicotinamide per day for two months can be expected to increase plasma tryptophan between 20% and 80%.
9. As a doctor, I recognize that no two patients are the same. I also recognize that different patients react differently to the same treatment. Patients react differently to the same treatment for a myriad of reasons including different diets, different stress levels, different metabolic capacities, and/or different genetic backgrounds.
10. If I were inclined to practice the invention disclosed in the application, I would:
- a. In the ordinary case, initiate treatment for a tryptophan depletion by orally administering a daily dose of nicotidimide in the preferred amount of 500 milligrams per day.
 - b. In a more extreme case of tryptophan depletion, I would initiate treatment by orally

administering a daily dose of nicotidimide in the preferred amount of 3 grams per day.

- c. In either case, I would re-assess the patient at a subsequent date. If the tryptophan levels had increased I would maintain the treatment until tryptophan level had returned to an appropriate level. If the tryptophan level had not increased, I would raise the dosage commensurate with the condition.
11. In summary, the application as a whole communicates to me how to use niacin to treat patients in need of an increase in systemic tryptophan.
12. In my opinion, no additional information or experimentation is needed for me to use niacin to treat patients in need of an increase in systemic tryptophan.
13. The potential utility of this pharmacologic approach in such patients may ultimately avoid damaging and life-threatening metabolic complications now experienced by these patients after long term anti-viral treatment.
14. It is true the application only provided four working examples, all at a dosage level of 3 grams/day, it is not unreasonable to extrapolate that information to arrive at appropriate starting dosages for other situations. In my opinion, these are matters which could be expected to be within the knowledge of someone who treats patients with retroviruses.
15. At most, I might want to determine the upper limit of tolerance for this medication approach (duration or dosage).
16. All statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true;
17. These statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. 1001, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

David G. Sidebottom, M.D.

David G. Sidebottom, M.D.

June 17, 2003

Date

**Treatment Of Retrovirus Induced Derangements With Niacin
Compounds**

5

FIELD OF INVENTION

This invention relates to the treatment of mammals chronically infected with
10 retroviruses, such as human immunodeficiency virus [HIV].

BACKGROUND

Retroviruses lead to chronic infection in mammals. Retroviruses are packets of
infectious nucleic acids (i.e. genetic material) surrounded by a protective protein coat.
15 Retroviruses are incapable of generating metabolic energy or synthesizing proteins, and
thus are characterized by dependence on living cells for replication and proliferation. A
retrovirus contains three enzymes: (1) reverse transcriptase, (2) protease, and (3)
integrase. Current antiviral drug therapy focuses on the inhibition of reverse transcriptase
and protease enzymes.

20 HIV is a prototypic retrovirus that causes the acquired immunodeficiency
syndrome [AIDS] in humans and related primates. Worldwide, AIDS has claimed over
11 million lives. HIV currently infects more than 30 million people. Since the first
reported cases of AIDS almost 20 years ago, the medical community has learned much
about this retroviral disease and its diverse manifestations. A number of clinical

manifestations of HIV infection, however, remain unexplained despite the efforts of the medical community to discover their etiology.

The Center for Disease Control and Prevention (the "CDC") has developed a "case definition" of the specific findings which, if present in a person with HIV, define
5 AIDS. See Center for Disease Control and Prevention, *1993 Revised Classification System for HIV Infection and Expanded Surveillance Case Definition for AIDS Among Adolescents and Adults*, MMWR Morb Mortal Wkly Rep, 41(RR-17): 1-19(1992). The CDC's case definition falls into three broad categories: (1) CD4 immune cell depletion, (2) opportunistic infections, and (3) malignancies.

10 In addition to the case definition of AIDS, a number of metabolic changes are associated with this chronic infection. Among them are alterations in the circulating concentrations of amino acids. Amino acids are often referred to as the building blocks of proteins. Of the common amino acids, ten amino acids are "essential." The essential amino acids are those which the body cannot synthesize and therefore must be obtained
15 directly through the diet.

Tryptophan, an essential amino acid, is known to be depleted during HIV infection. The body utilizes dietary-derived tryptophan for several important biochemical functions, including: (1) as a building block in the synthesis of proteins, (2) as a precursor of niacin and nicotinamide adenine dinucleotide [NAD], and (3) as a
20 precursor of serotonin. Attempting to simply replete plasma tryptophan directly through pharmacologic doses of tryptophan is not advisable given the history of patients developing "eosinophilia myalgia syndrome."

Chronic retroviral infections lead to an ongoing metabolic burden on the infected subject. This burden in HIV infection includes: (1) the turnover of CD4 cells, (2) the disturbance of lipid metabolism, (3) the depletion of serotonin, (4) the depletion of plasma tryptophan [as discussed above], and (5) the depletion of intracellular NAD. The infection, over the course of months, leads to immunodeficiency (marked by CD4 depletion) and opportunistic infections. The infection also leads to a metabolic disease state marked by a number of other manifestations, including a non-specific "wasting syndrome" and the specific disturbances and depletions previously mentioned in this paragraph.

Presently, no cure exists for HIV infection. Current treatments for HIV infected patients tend to focus on agents which inhibit two viral enzymes: the HIV-reverse transcriptase [reverse transcriptase inhibitors] or the HIV-protease [protease inhibitors]. Such agents include among others, ZDV (zidovudine), DDI (2'-3' -dideoxyinosine), and DDC (2' -3' -dideoxycytidine), each of which blocks the HIV proliferation in cells (ZDV, DDI, DDC and other such agents are referred to as the "licensed antivirals").

Unfortunately, the inhibition which occurs with the licensed antivirals is incomplete. Over time, HIV becomes resistant to the licensed antivirals. This resistance can result in a resumption of progressive immune system destruction.

Zidovudine, a licensed antiviral compound, is the only compound known to replete plasma tryptophan in HIV infected persons. However, zidovudine which is a reverse transcriptase inhibitor, causes a number of side effects including headache, nausea, and bone marrow suppression. Furthermore, HIV can develop resistance to

Zidovudine, an event which would be expected to result in recurrent tryptophan depletion.

Since HIV depletes plasma tryptophan and since this essential amino acid is required in a range of biologically necessary tasks, replenishing plasma tryptophan is essential in maintaining overall health in the HIV infected state. Although the antiviral drug zidovudine leads to an increase in plasma tryptophan in HIV infected persons, this reversal would be expected to last only so long as virus inhibition persists, and antiviral drug failure is expected with time given the incomplete nature of the drug's inhibitory effect. Niacin, as an agent to reverse infection-induced metabolic changes, works on the host side of the virus-host interaction and therefore would not be subject to the same risk of eventual viral drug resistance.

BRIEF SUMMARY OF THE INVENTION

This invention inhibits adverse metabolic and immunologic effects associated with chronic retroviral infections such as HIV by using niacin compounds, such as nicotinamide or nicotinic acid, to inhibit the depletion of tryptophan and to induce the restoration of intracellular nicotinamide nucleotides, such as nicotinamide adenine dinucleotide [NAD], in patients with retroviral infections.

More particularly, this invention relates to the oral use of pharmacologic doses of niacin compounds in persons with HIV infection in order to reverse or prevent deleterious metabolic consequences of the infection.

Another object of the invention is to inhibit adverse effects of HIV infection by combining the method of this invention with known HIV inhibitors, such as reverse transcriptase inhibitors, protease inhibitors, and others.

The invention provides a method of administering a therapeutically effective
5 amount of niacin compounds to a patient with a chronic retroviral infection such as HIV, the etiological agent clinically associated with AIDS.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

Table 1 - Baseline Characteristics of Niacin Study Patients. Illustrates the
10 immunological status as measured by CD4 count, the concomitant use of antiviral medications, and the presence of co-infections. Niacin worked to improve tryptophan status in all four patients across this range of baseline infectious disease related findings.

Table 2 - Baseline Dietary intake of Niacin Study Patients. Illustrates the range of
baseline dietary intake of tryptophan and niacin compounds. The amounts were
15 determined by dietary recall survey, and demonstrate that tryptophan and niacin were not deficient in the baseline diet of these patients, and that the pharmacological dose of niacin used in the study was significantly higher than all participant's baseline intake.

Table 3 - Changes in plasma tryptophan levels [micromols/l] in patients taking 3
gram of nicotinamide daily for 2 months. The increase in the levels of this essential
20 amino acid despite the unchanged dietary intake of tryptophan is consistent with decreased metabolic shunting of essential tryptophan towards niacin in HIV infected persons.

Table 4 - Changes in non-tryptophan plasma amino acid levels in HIV patients taking 3 grams/day of oral nicotinamide. The four amino acids include two essential amino acids [methionine and lysine] and two nonessential amino acids [cysteine and taurine]. In all four cases there is no discernible pattern of change with this intervention, supporting the observation that the effect of pharmacological doses of niacin on plasma tryptophan is a specific and important intervention against the metabolic disruption caused by HIV infection.

DESCRIPTION

The invention is a method for treatment of HIV infected persons with niacin administered in an amount effective to combat plasma tryptophan depletion. This invention is useful for any mammal infected with a retrovirus, including HIV. Through administration of a pharmacological dose of niacin, the retrovirus-infected subject's systemic tryptophan depletion will be reversed.

Niacin refers to either of two chemically related compounds: nicotinamide or nicotinic acid. Niacin may be administered orally, parenterally, rectally, or with any pharmaceutically accepted adjuvant or carrier. The administration and effects of niacin have undergone extensive study in the fields of diabetes and hypercholesterolemia. (*See, e.g., Petley A, et al, The Pharmacokinetics of Nicotinamide in Humans and Rodents, Diabetes*, 44: 152-155 (1995); and DiPalma JR and Thayer WS, *Use of Niacin as a Drug*, *Annu. Rev. Nutr.*, 11:169-87, (1991)). Niacin, or vitamin B3, is the common name for both nicotinic acid, i.e., C₆H₅N₂O₂, (pyridine-3-carboxylic acid) or nicotinamide, i.e., C₆H₆N₂O₂ (3-pyridinecarboxamide).

Niacin is a precursor to the biosynthesis of nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP). Nicotinamide nucleotides (NAD and NADP) participate in a wide array of oxidation-reduction reactions catalyzed by dehydrogenase or oxido-reductase enzymes. Virtually every aspect of cellular metabolism involves NAD/NADH or NADP/NADPH dependent reactions. In absence of sufficient supplies of nicotinamide nucleotides or niacin precursors for nicotinamide nucleotide biosynthesis, cellular functions and life itself would be impaired. (DiPalma JR and Thayer WS, *Use of Niacin as a Drug*, Annu. Rev. Nutr., 11:169-87, (1991)). The body can readily convert nicotinic acid to nicotinamide and both are expected to produce the desired therapeutic effect of combating plasma tryptophan depletion.

For this invention, it is preferred to administer niacin in "pharmacologic doses." A vitamin compound is considered a "drug," not a "nutrient," when: [1] the ingested dose exceeds the dose required for nutrient function, and [2] a pharmacologic action distinct from nutrient function is achieved. Maintaining plasma tryptophan is not a nutrient function of niacin; rather, it is a pharmacological action of niacin in retrovirally infected subjects.

All vitamins fill a nutrient function whereby a sufficient amount of the vitamin compound is required in the diet to fulfill normal metabolic needs. The body normally requires 12-18 milligrams of niacin per day to carry out the coenzyme function which defines niacin as a vitamin. The Recommended Daily Allowance [RDA] of niacin is approximately 13-20 milligrams per day. Therefore, a non-pharmacologic dose of niacin,

where niacin acts as a vitamin or nutrient compound, is approximately 20 milligrams a day or less.

The use of pharmacologic doses of niacin is distinct from the vitamin or nutrient use of niacin. (DiPalma JR and Thayer WS, *Use of Niacin as a Drug*, Annu. Rev. Nutr., 5 11:169-87, (1991)). Niacin's pharmacologic use can be distinguished from its non-pharmacologic (or physiologic) use by the pharmacodynamic action of the compound. Pharmacodynamic action begins when the nutrient function of niacin is complete. The maintenance of plasma tryptophan in the face of (1) retrovirus infection, and (2) normal or supernormal niacin levels is the distinct pharmacodynamic action described here.

10 A pharmacological dose of niacin generally occurs at a dose of about 100 milligrams per day, about 5 times the recommended daily allowance [RDA]. Niacin is safe in doses greater than 100 mg in persons with HIV, and doses of greater than 100 mg should also cause a retrovirus-infected patient to undergo a reverse systemic tryptophan depletion.

15 Because pharmacologic doses of niacin alleviate the drive to deplete plasma tryptophan, tryptophan depletion may represent a metabolic shunt towards niacin production. (See Murray, *Niacin as a Potential AIDS Preventative Factor*, Medical Hypotheses 53(5), 375-379 (November 1999), which is incorporated herein by reference.) In addition, because the essential amino acid tryptophan cannot be synthesized in the 20 body, any agent which increases in the circulating concentrations of tryptophan in HIV infected persons presumably does so by diminishing the metabolic demands on the available supply.

The preferred embodiment of this invention is to administer a mammal infected with a retrovirus with niacin. The preferred method of administration is oral administration. The preferred dose is 500 milligrams of niacin per day in the form of nicotinamide.

5 The following EXAMPLE is presented to more fully illustrate the preferred embodiment of the invention. The example should not be construed to limit the scope of the invention and is to be understood merely for the purpose of illustration.

EXAMPLE - Clinical Trial of Niacin in HIV infected persons.

10 Four HIV infected persons participated in a trial of niacin in the form of nicotinamide. The participants were at various stages of their HIV infection as judged by their CD4 counts which ranged from 0 to 620 [see table 1]. The participants were receiving either a stable regimen of anti-viral drugs [i.e. anti-HIV drugs] for a period greater than one year or were not taking any anti-viral drugs. Two of the participants had known co-infections typical of HIV
15 infected persons. Each participant took 3 grams of nicotinamide per day for 2 months. This treatment was not associated with any adverse side effects. Each participant's plasma tryptophan was measured prior to treatment and at the end of treatment [see table 3]. The average increase of plasma tryptophan of all participants was 43.9%. This change in tryptophan concentration was statistically
20 significant with a calculated p value of $p=0.0112$ [using paired t-test]. The study also measured 4 other plasma amino acids which are listed in table 4. All amino acid concentrations were measured by High Performance Liquid Chromatography [HPLC]. There was no significant change in the plasma amino acid

concentrations other than tryptophan. As demonstrated in tables 3 and 4, only plasma tryptophan changed in a statistically significant manner.

The details of the invention have been set forth in the accompanying description and example above. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials have been described. Other features, object, and advantages of the invention will be apparent from the description and from the claims. In the specification and the claims, the singular forms include plural referents unless the context clearly requires otherwise. Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. All patents and publications cited in this specification are incorporated by reference.

CLAIMS

What is claimed is:

1. A method for treating a patient infected with a retrovirus, which comprises the step of administering a daily pharmacological dose of niacin.
- 5 2. A method for treating retrovirus-induced metabolic changes, which comprises the step of administering a daily pharmacological dose of niacin.
3. A method for treating a patient infected with HIV, which comprises the step of administering a daily pharmacological dose of niacin.
- 10 4. A method for treating HIV-induced metabolic changes, which comprises the step of administering a daily pharmacological dose of niacin.
5. A method for treating retrovirus-induced metabolic changes in a patient's systemic tryptophan levels, which comprises the step of administering a daily pharmacological dose of niacin.
- 15 6. A method for treating HIV-induced metabolic changes in a patient's systemic tryptophan levels, which comprises the step of administering a daily pharmacological dose of niacin.
7. A method for treating the depletion of tryptophan in a retrovirus-infected patient, which comprises the step of administering a daily pharmacological dose of niacin.
- 20 8. A method for treating the depletion of tryptophan in an HIV-infected patient, which comprises the step of administering a daily pharmacological dose of niacin.

9. A method for replenishing nicotinamide nucleotide precursors, which comprises the step of administering a daily pharmacological dose of niacin.
10. The method of claim 1, 2, 3, 4, 5, 6, 7, 8, or 9, where said dose is of an amount sufficient to prevent retrovirus-induced metabolic changes in systemic tryptophan concentrations.
11. The method of claim 1, 2, 3, 4, 5, 6, 7, 8, or 9, where said dose is of an amount sufficient to slow down the rate of retrovirus-induced metabolic changes in systemic tryptophan concentrations.
12. The method of claim 1, 2, 3, 4, 5, 6, 7, 8, or 9, where said dose is of an amount sufficient to stop the rate of retrovirus-induced metabolic changes in systemic tryptophan concentrations.
13. The method of claim 1, 2, 3, 4, 5, 6, 7, 8, or 9, where said dose is of an amount sufficient to increase a patient's level of plasma tryptophan.
14. A method as in claim 1, 2, 3, 4, 5, 6, 7, 8, or 9, where said dose is greater than 100 milligrams per day.
15. A method as in claim 1, 2, 3, 4, 5, 6, 7, 8, or 9, where said dose is approximately 3 grams per day.
16. A method as in claim 1, 2, 3, 4, 5, 6, 7, 8, or 9, where said dose exceeds the standard recommended daily amounts for coenzyme activity.
17. A method of claim 1, 2, 3, 4, 5, 6, 7, 8, or 9, where said dose exceeds amounts normally obtainable with routine diet and supplement practices.

18. A method as in claim 1, 2, 3, 4, 5, 6, 7, 8, or 9, where said dose exceeds the RDA [recommended daily allowance] of niacin.
19. A method as in claim 1, 2, 3, 4, 5, 6, 7, 8, or 9, where said dose is sufficient to raise the intracellular levels of nicotinamide adenine dinucleotide [NAD] in persons with HIV infection.
20. A method as in claim 1, 2, 3, 4, 5, 6, 7, 8, or 9, where said dose is sufficient to replete nicotinamide nucleotide precursors [NAD].
21. A method of claim 1, 2, 3, 4, 5, 6, 7, 8, or 9, where said dose of niacin is administered to persons with HIV and other co-infections.
22. A method as in claim 1, 2, 3, 4, 5, 6, 7, 8, or 9, where said dose of niacin is administered in combination with antiviral medications such as reverse transcriptase inhibitors, and protease inhibitors.
23. A method as in claim 1, 2, 3, 4, 5, 6, 7, 8, or 9, where said dose is administered in combination with other treatments for HIV infection to improve the metabolic status of an infected patient.
24. A method as in claim 1, 2, 3, 4, 5, 6, 7, 8, or 9, where said dose is sufficient to inhibit new virus production.

ABSTRACT OF THE DISCLOSURE

Chronic infection with retroviruses, such as HIV, induce a number of metabolic derangements. The present invention relates to a method for treating retrovirus-infected subjects with niacin compounds to reverse infection induced metabolic derangements.

5

DRAWINGS

Table 1 - Baseline Infectious Disease Characteristics of Nicotinamide Study Patients.

5

Patient	CD4 count	Antiretroviral [duration]	Co-infections
1	0	none	molluscum contagiosum
2	220	PI ¹ /RTI ² [3 years]	none
3	290	RTI [2 years]	none
4	620	none	herpes zoster

**Table 2 - Baseline Dietary Characteristics of Nicotinamide Study Patients. Daily intake
for tryptophan and niacin by dietary survey**

10 **[i.e. these numbers reflect the total non-pharmacologic amounts included in participants
food and nutritional supplements.]**

Patient	Tryptophan [daily intake]	Niacin [RDA %]
1	0.89 gms	42.0 mg [210%]
2	1.44 gms	22.4 mg [112%]
3	0.66 gms	32.8 mg [164%]
4	1.05 gms	24.0 mg [120%]

15

¹ PI is protease inhibitor.

² RTI is reverse transcriptase inhibitor.

Table 3 - Changes in plasma tryptophan levels [micromols/l] in patients taking 3 gram of nicotinamide daily for 2 months.

Patient	Days of Treatment	Baseline Plasma Tryptophan	Final Plasma Tryptophan	Change in Plasma Tryptophan
1.	57	31.1	52.9	+ 70.1%
2.	61	53.4	82.3	+ 54.1 %
3.	63	62.0	75.1	+ 21.1%
4.	60	51.0	66.5	+ 30.4%

Table 4 - Changes in non-tryptophan plasma amino acid levels in HIV infected patients taking 3 grams/day of oral nicotinamide.

Patient	Days of Treatment	Baseline Plasma Methionine	Final Plasma Methionine	Change in Plasma Methionine
1.	57	19.8	18.3	- 7.6%
2.	61	15.6	17.1	+ 9.6 %
3.	63	34.3	24.4	- 28.9%
4.	60	18.3	20.4	+ 11.5%

5

Patient	Days of Treatment	Baseline Plasma Lysine	Final Plasma Lysine	Change in Plasma Lysine
1.	57	218.7	111.1	- 49.2%
2.	61	97.7	141.2	+ 44.5 %
3.	63	251.8	162.7	- 34.5%
4.	60	191.8	129.1	- 32.7%

Patient	Days of Treatment	Baseline Plasma Cysteine	Final Plasma Cysteine	Change in Plasma Cysteine
1.	57	48.3	54.7	+ 13.3%
2.	61	27.0	28.8	+ 6.6 %
3.	63	35.6	39.1	+ 9.8%
4.	60	75.5	61.3	-18.8%

Table 4 (cont.)

Patient	Days of Treatment	Baseline Plasma Taurine	Final Plasma Taurine	Change in Plasma Taurine
1.	57	46.3	68.8	+ 48.6%
2.	61	76.2	87.4	+ 14.4 %
3.	63	92.1	69.7	- 24.3%
4.	60	80.6	61.6	- 23.6%

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